428 Rec'd PCT/PTO 03 DEC 1999

FORM PTO-1390 (REV 10-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER: FHW-051US

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C.371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/744528

INTERNATIONAL APPLICATION	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB98/01619	**03 June 1998 (03.06.98)**	**04 June 1997 (04.06.97)**

TITLE OF INVENTION

BACTERIAL PHEROMONES AND USES THEREFOR

APPLICANT(S) FOR DO/EO/US

Galina V. MUKAMOLOVA; Arseny S. KAPRELYANTS; Danielle I. YOUNG; Douglas B. KELL; and Michael YOUNG

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C.371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371 (b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau)
   (72 sheets and 20 sheets of drawings);
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) **(unexecuted) (4 sheets);**
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included
13. ☒ A FIRST preliminary amendment **(9 sheets);**
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information: **Transmittal Letter (2 sheets in duplicate); PCT Request (Form PCT/R/101) (6 sheets); PCT International Published Application (WO 98/55624) (with International Search Report attached); (75 sheets); PCT Notification of Transmittal of the International Preliminary Examination Report (Form PCT/IPEA/416) (8 sheets); Sequence Listing (31 sheets); Sequence Listing Diskette; Transmittal Letter for Diskette of Sequence Listing (1 sheet); Certificate of Express Mailing (1 sheet); and Return Postcard.**

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NO.
09/445289	PCT/GB98/01619	FHW-051US

17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) .(a/o November 1, 1999):		
Search Report has been prepared by the EPO or JPO $840		
International preliminary examination fee paid to USPTO (37 CFR 1.482) $670		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $760		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $970		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $96		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$840	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	65 -20 =	45	X $18.00	$810	
Independent claims	21 -3 =	18	X $78.00	$1404	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ 260.00	$--	
TOTAL OF ABOVE CALCULATIONS =				$3054	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28)				$--	
SUBTOTAL =				$3054	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$--	
TOTAL NATIONAL FEE =				$3054	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$--	
TOTAL FEES ENCLOSED =				$--	
				Amount to be: refunded	$
				charged	$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **12-0080** in the amount of **$3054** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **12-0080**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Anthony A. Laurentano, Esq.
LAHIVE & COCKFIELD, LLP
28 State Street
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Date: 03 December 1999

SIGNATURE

Peter C. Lauro
NAME

32,360
REGISTRATION NUMBER

#3

Applicant or Patentee: Galina V. MUKAMOLOVA et al.
Serial or Patent No.: U.S. National Phase of PCT/GB98/01619
Filed or Issued: 03 June 1998
Title: BACTERIAL PHEROMONES AND USES THEREFOR

Attorney's Docket No.: FHW-051US

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF NONPROFIT ORGANIZATION THE UNIVERSITY OF WALES
ADDRESS OF NONPROFIT ORGANIZATION Aberystwyth, Old College, King Street, Aberystwyth SY23 2AX
TYPE OF NONPROFIT ORGANIZATION

- ☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
- ☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
- ☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
  (NAME OF STATE _____)
  (CITATION OF STATUTE _____)
- ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
- ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
  (NAME OF STATE _____)
  (CITATION OF STATUTE _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in

- ☐ the specification filed herewith with title as listed above.
- ☒ the application identified above.
- ☐ the patent identified above.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization regarding the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____
ADDRESS _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME _____
ADDRESS _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Keith LEWIS

TITLE IN ORGANIZATION OF PERSON SIGNING DIRECTOR of FINANCE

ADDRESS OF PERSON SIGNING UNIVERSITY OF WALES ABERYSTWYTH, OLD COLLEGE, KING STREET, ABERYSTWYTH SY23 2AX UK

X _____ 14 JANUARY 2000
SIGNATURE DATE

IN THE UNITED STATES PATENT DESIGNATED OFFICE (DO/US)
(National Phase of International App.: PCT/GB98/01619)

In re the application of: Galina V. Mukamolova, *et al.*

Serial No.: Not Assigned

Filed: Herewith

For: *BACTERIAL PHEROMONES AND USES THEREFOR*

Attorney Docket No.: FHW-051US

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATION UNDER 37 CFR 1.10

Date of Deposit: December 3, 1999 Mailing Label Number: EL263573297 US

I hereby certify that this 37 CFR 1.53(b) request and the documents referred to as attached therein are being deposited with the United States Postal Service on the date indicated above in an envelope as "Express Mail Post Office to Addressee" service under 37 CFR 1.10 and addressed to the Assistant Commissioner for Patents, Box Patent Application, Washington, D.C. 20231.

Deise K. Timas
Name of Person Mailing Paper

Deise K Timas
Signature of Person Mailing Paper

PRELIMINARY AMENDMENT

Dear Sir or Madam:

Prior to examination, please amend the above-referenced application as follows:

In the Claims:

Please cancel claims 1-60 without prejudice.

Please add the following new claims:

61. An isolated polypeptide capable of resuscitating dormant, moribund or latent bacterial cells, which polypeptide comprises: (i) a sequence of amino acid residues wherein the identities and relative positions of amino acid residues therein correspond to the residues indexed by asterisks in any one of the sequences set out in Figure 1 A or Figure 1 B(B), or (ii) a sequence which has at least 20% identity or homology with the sequence defined in (i).

62. The polypeptide of claim 61 which is any one of the polypeptides represented in Figure 1 A or Figure 1 B, or a homologue, allelic form, species variant or mutein thereof.

63. The polypeptide of claim 61 which is the *M. luteus* Rpf factor represented in Fig. 2A, or a homologue, allelic form, species variant or mutein thereof.

64. The polypeptide of claim 61 which is recombinant.

65. A pharmaceutical composition (e.g. a vaccine) comprising the polypeptide of claim 61.

66. The polypeptide of claim 61 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

67. An antibody (or antibody derivative) specific for the polypeptide of claim 61.

68. The antibody of claim 67 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

69. Isolated nucleic acid encoding the polypeptide defined in claim 61.

70. A vector (e.g. an expression vector) comprising the nucleic acid of claim 69.

71. A host cell comprising the vector of claim 70.

72. The nucleic acid of claim 69 or vector of claim 70 in a pharmaceutical excipient.

73. A diagnostic kit, culture medium or transport medium comprising the polypeptide of claim 61.

74. An ex *vivo* method of diagnosis, comprising the step of contacting a biological sample with the polypeptide of claim 61.

75. A live vaccine comprising an attenuated microbe, which microbe bears a mutation in a gene encoding (or regulating the expression of) the polypeptide of claim 61.

76. An isolated nucleic acid molecule selected from the group consisting of:

(a) a nucleic acid molecule comprising the nucleotide sequence set forth in SEQ ID NO: 35; and

(b) a nucleic acid molecule comprising the nucleotide sequence set forth in SEQ ID NO: 54.

77. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 36.

78. An isolated nucleic acid molecule which encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 36.

79. An isolated nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule comprising a nucleotide sequence which selectively or specifically cross hybridizes with the nucleotide sequence of SEQ ID NO: 35 or 54, or a complement thereof;

b) a nucleic acid molecule comprising a fragment of at least 150 nucleotides of a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 35 or 54, or a complement thereof;

c) a nucleic acid molecule which encodes a polypeptide comprising an amino acid sequence at least about 20% homologous to the amino acid sequence of SEQ ID NO: 2; and

d) a nucleic acid molecule which encodes a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO: 36, wherein the fragment comprises at least 15 contiguous amino acid residues of the amino acid sequence of SEQ ID NO: 36.

80. An isolated nucleic acid molecule which hybridizes to the nucleic acid molecule of any one of claims 76, 77, 78, or 79 under stringent conditions.

81. An isolated nucleic acid molecule comprising a nucleotide sequence which is complementary to the nucleotide sequence of the nucleic acid molecule of any one of claims 76, 77, 78, or 79 .

82. An isolated nucleic acid molecule comprising the nucleic acid molecule of any one of claims 76, 77, 78, or 79 , and a nucleotide sequence encoding a heterologous polypeptide.

83. A vector comprising the nucleic acid molecule of any one of 76, 77, 78, or 79 .

84. The vector of claim 82, which is an expression vector.

85. A host cell transfected with the expression vector of claim 84.

86. A method of producing a polypeptide comprising culturing the host cell of claim 85 in an appropriate culture medium to, thereby, produce the polypeptide.

87. An isolated polypeptide selected from the group consisting of:

a) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO: 36, wherein the fragment comprises at least 15 contiguous amino acids of SEQ ID NO: 36;

b) a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO: 36, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule consisting of SEQ ID NO: 35 or 54 under stringent conditions;

c) a polypeptide which is encoded by a nucleic acid molecule comprising a nucleotide sequence which selectively or specifically cross hybridizes to a nucleic acid comprising the nucleotide sequence of SEQ ID NO: 35 or 54; and

d) a polypeptide comprising an amino acid sequence which is at least 20% homologous to the amino acid sequence of SEQ ID NO: 36.

88. The isolated polypeptide of claim 87 comprising the amino acid sequence of SEQ ID NO: 36.

89. The polypeptide of claim 87, further comprising heterologous amino acid sequences.

90. An antibody which selectively binds to a polypeptide of claim 87.

91. A method for detecting the presence of a polypeptide of claim 87 in a sample comprising:

a) contacting the sample with a compound which selectively binds to the polypeptide; and

b) determining whether the compound binds to the polypeptide in the sample to thereby detect the presence of a polypeptide of claim 87 in the sample.

92. The method of claim 91, wherein the compound which binds to the polypeptide is an antibody.

93. A kit comprising a compound which selectively binds to a polypeptide of claim 87 and instructions for use.

94. A method for detecting the presence of a nucleic acid molecule of any one of claims 76, 77, 78, or 79 in a sample comprising:

a) contacting the sample with a nucleic acid probe or primer which selectively hybridizes to the nucleic acid molecule; and

b) determining whether the nucleic acid probe or primer binds to a nucleic acid molecule in the sample to thereby detect the presence of a nucleic acid molecule of any one of claims 76, 77, 78, or 79 in the sample.

95. The method of claim 94, wherein the sample comprises mRNA molecules and is contacted with a nucleic acid probe.

96. A kit comprising a compound which selectively hybridizes to a nucleic acid molecule of any one of claims 76, 77, 78, or 79 and instructions for use.

97. A method for identifying a compound which binds to a polypeptide of claim 87 comprising:

a) contacting the polypeptide, or a cell expressing the polypeptide with a test compound; and

b) determining whether the polypeptide binds to the test compound.

98. The method of claim 97, wherein the binding of the test compound to the polypeptide is detected by a method selected from the group consisting of:

a) detection of binding by direct detection of test compound/polypeptide binding;

b) detection of binding using a competition binding assay; and

c) detection of binding using an assay for RP-factor activity.

99. A method for modulating the activity of a polypeptide of claim 87 comprising contacting the polypeptide or a cell expressing the polypeptide with a compound which binds to the polypeptide in a sufficient concentration to modulate the activity of the polypeptide.

100. A method for identifying a compound which modulates the activity of a polypeptide of claim 87 comprising:

a) contacting a polypeptide of claim 87 with a test compound; and

b) determining the effect of the test compound on the activity of the polypeptide to thereby identify a compound which modulates the activity of the polypeptide.

101. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 1.

102. The isolated polypeptide of claim 101 comprising the amino acid sequence of SEQ ID NO: 1.

103. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 2.

104. The isolated polypeptide of claim 103 comprising the amino acid sequence of SEQ ID NO: 2.

105. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 3.

106. The isolated polypeptide of claim 105 comprising the amino acid sequence of SEQ ID NO: 3.

107. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 4.

108. The isolated polypeptide of claim 107 comprising the amino acid sequence of SEQ ID NO: 4.

109. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 5.

110. The isolated polypeptide of claim 109 comprising the amino acid sequence of SEQ ID NO: 5.

111. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 6.

112. The isolated polypeptide of claim 111 comprising the amino acid sequence of SEQ ID NO: 6.

113. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 7.

114. The isolated polypeptide of claim 113 comprising the amino acid sequence of SEQ ID NO: 7.

115. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 8.

116. The isolated polypeptide of claim 115 comprising the amino acid sequence of SEQ ID NO: 8.

117. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 9.

118. The isolated polypeptide of claim 117 comprising the amino acid sequence of SEQ ID NO:9.

119. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 10.

120. The isolated polypeptide of claim 119 comprising the amino acid sequence of SEQ ID NO:10.

121. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 11.

122. The isolated polypeptide of claim 121 comprising the amino acid sequence of SEQ ID NO: 11.

123. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 12.

124. The isolated polypeptide of claim 123 comprising the amino acid sequence of SEQ ID NO: 12

125. A pharmaceutical composition comprising a polypeptide and a pharmaceutically acceptable carrier, said polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, and 36.--

REMARKS

Claims 1-60 were originally present in the application. Claims 1-60 have now been canceled and new claims 61-125 have been added. Accordingly, claims 61-125 are currently pending in the application.

Claims 61-75 have been substantially copied from the parent application PCT/GB98/01619 by Mukamolova *et al.*, filed on June 4, 1998, published as WO 98/55624 on December 10, 1998, which is incorporated by reference in its entirety in the present application. Accordingly, claims 61-64 find support in claims 1-13 and 15-18; claims 65-66 find support in claims 19 and 20; claims 67-68 find support in claims 13, 14, and 21-22; and claims 69-75 find support in claims 30-32, 35, 42, and 57.

Additional claims 76 to 125 also find support in the specification and the claims as originally filed. In particular, claims 76-82 find support, for example, in claims 1, 30, and Fig. 2A as originally filed. In addition, claims 83-86 find support in originally filed claims 31, 32, and 45; claims 87-89 find support in, for example, originally filed claims 1-12 and 15-18; claims 90-95 find support in, for example, originally claims 15, 37-38, and 40; claims 96-100 find support in, for example, originally filed claims 34-35 and 37-39; and claims 101-125 find support in, for example, claims 5 and 30, as originally filed, and Figs. 1A and 1B of the specification.

No new matter has been added. Applicants request that the new claims be entered.

If a telephone conversation with Applicants' Attorney would expedite the prosecution of the above-identified application, the examiner is urged to call Applicants' Attorney at (617) 227-7400.

Respectfully submitted,

Peter C. Lauro, Esq.
Registration No. 32,360
Attorney for Applicants

LAHIVE & COCKFIELD, LLP
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Tel. (617) 227-7400
Dated: December 3, 1999

CLAIMS:

1. An isolated polypeptide capable of resuscitating dormant, moribund or latent bacterial cells, which polypeptide comprises: (i) a sequence of amino acid residues wherein the identities and relative positions of amino acid residues therein correspond to the residues indexed by asterisks in any one of the sequences set out in Figure 1A or Figure 1B(B), or (ii) a sequence which has at least 20% identity or homology with the sequence defined in (i).

2. The polypeptide of claim 1 which is any one of the polypeptides represented in Figure 1A or Figure 1B, or a homologue, allelic form, species variant or mutein thereof.

3. The polypeptide of claim 1 which is the *M. luteus* Rpf factor represented in Fig. 2A, or a homologue, allelic form, species variant or mutein thereof.

4. The polypeptide of any one of the preceding claims which is recombinant.

5. A pharmaceutical composition (e.g. a vaccine) comprising the polypeptide of any one of the preceding claims.

6. The polypeptide of any one of claims 1 to 4 which is:

(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or

(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

7. An antibody (or antibody derivative) specific for the polypeptide of any one of claims 1 to 4.

8. The antibody of claim 7 which is:

(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or

(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

9. Isolated nucleic acid encoding the polypeptide defined in any one of claims 1 to 4.

10. A vector (e.g. an expression vector) comprising the nucleic acid of claim 9.

11. A host cell comprising the vector of claim 10.

12. The nucleic acid of claim 9 or vector of claim 10 in a pharmaceutical excipient.

13. A diagnostic kit, culture medium or transport medium comprising the polypeptide of any one of claims 1 to 4.

14. An *ex vivo* method of diagnosis, comprising the step of contacting a biological sample with the polypeptide of any one of claims 1 to 4.

15. A live vaccine comprising an attenuated microbe, which microbe bears a mutation in a gene encoding (or regulating the expression of) the polypeptide defined in any one of claims 1 to 4.

BACTERIAL PHEROMONES AND USES THEREFOR

Related Information

This application corresponds to international patent application PCT/GB98/01619, filed June 3, 1998, and published as WO 98/55624 on December 10, 1998, the disclosure of which is incorporated herein in its entirety by reference.

Field of the invention

The present invention relates to RP-factors, their cognate receptors, convertases, respective genes and to inhibitors or mimetics thereof. In particular, the invention relates to antibodies, pharmaceutical compositions and (therapeutic, diagnostic) methods based on the RP-factors and their receptors/convertases.

Introduction

Bacterial pheromones

It is known that certain chemicals may mediate intercellular communication in bacterial cultures. Such communication has been shown to be of importance during sporulation, conjugation, changes in virulence and in bioluminescence. It is now clear that a variety of different autocrine and/or paracrine chemical compounds ("pheromones") produced as secondary metabolites are responsible for such social behaviour in prokaryotes (see e.g. Kell et al., 1995, Trends Ecol. Evolution, 10, 126-129).

Pheromones may be distinguished from nutrients inter alia in that: (i) they are produced by the organisms themselves, (ii) they are active at very low concentrations (e.g. at picomolar or nanomolar concentrations), and (iii) with the exception of prohormone processing, their *metabolism* is not necessary for activity (although they may of course ultimately be degraded).

The chemical nature of these pheromonal compounds varies widely: those associated with Gram-negative organisms tend to be of low molecular weight (e.g. N-acyl homoserine lactone derivatives), whilst a number of Gram-positive organisms use proteins and polypeptides (Kell et al,1995, *ibidem*).

Pheromones are also known to play an important role in the development of bacterial cultures. For unstressed (uninjured) bacteria and optimal growth media, the "self-promoting" mode of culture growth is normally masked due to the high rate of production of growth factors and the sensitivity of the cells to these pheromones. Only under unfavourable conditions (for example, poor growth media, small initial inocula and/or starved cells) is this self-promoting behaviour "visible".

For example, a dramatic reduction in the length of the lag phase of cultures of *Nitrosomonas europea* is mediated by N-(3-oxo-hexanoyl) homoserine lactone, and chorionic gonadotropin-like ligand (a 48kD protein) had similar growth-stimulating activity for *Xanthomonas maltophila*. A number of mammalian hormones (including peptide and steroid hormones as well as cytokines) have also been shown to exhibit potent growth-stimulating activities for both Gram-positive and Gram-negative bacteria.

Latency and resuscitation

The ability of a microbial cell to grow and divide on a nutrient agar plate constitutes the benchmark method for determining the number of living cells in a sample of interest. However, it is also widely recognised that, especially in nature, the distinction between life and non-life is not absolute; many cells may exist in "dormant" or "moribund" forms or states and will not produce colonies on nutrient media (i.e. are "non-culturable"). However, these dormant or latent cells are not dead: they can be returned, by a process known as resuscitation, to a state of viability/ culturability.

For example, it is known that cells of the (high-G+C Gram-positive) bacterium *Micrococcus luteus* can enter a state of true dormancy from which they may be resuscitated by culture supernatants, even in the absence of any 'initially viable' cells.

The latent state has profound medical implications: many pathogenic bacteria (including pathogenic mycobacteria such as *M. tuberculosis*) are known to persist for extended periods in latent states in a host organism. Indeed, tuberculosis is a re-emergent infection of great concern, and it is recognised in particular that the causative organism (*Mycobacterium tuberculosis*) can lie dormant (remain latent) in patients and carriers for periods of years.

The latent state also has important commercial implications, since it complicates many laboratory methods for the detection, cultivation and enumeration of bacteria (for example in the food and healthcare industries).

There is therefore a pressing need to understand the physiological bases of latency and resuscitation.

Summary of the invention

The present invention is based, at least in part, on the discovery of a new class of pheromones which stimulate the resuscitation of bacteria after true dormancy. This "resuscitation factor" (herein embraced by the term "RP-factor") may exhibit activity at picomolar concentrations (implying a non-nutritional role). The elucidation of the structure of the pheromones at the amino acid sequence level has also permitted the present inventors to describe a larger family of proteins, some members of which act more broadly as regulators of cellular growth or replication and not necessarily as resuscitation promoting factors. Further sequence comparisons have also led to the identification of the cognate receptors, at least some of which share certain sequence similarities with their cognate RP-factors.

Thus, in a first aspect of the present invention there is provided an isolated RP-factor.

RP-factors

The term "RP-factor" is used herein to encompass any representative of that family of substances the members of which are capable of resuscitating dormant, moribund or latent cells (e.g. dormant bacterial cells). In addition, the RP-factors of the invention may also exhibit growth-stimulatory activity with respect to growing cells (e.g. growing bacterial cells), and/or may be competent to reduce the lag time of cell (e.g. bacterial cell) cultures. The resuscitation activity (and optionally also the growth-stimulatory activity or lag-time reducing activity) of the RP-factor may be specific for a particular (bacterial) cell (e.g. specific for one or more pathogenic mycobacteria), or may be non-specific. Specificity may be manipulated for example by engineering (e.g. by mutagenesis or chimaerisation, as herein described) of the specificity-determining domain(s) of the RP-factor or by replacement of the signalling domain.

The term "RP-factor" is also used herein in a somewhat broader sense to encompass polypeptides which are expressed by bacteria and which regulate (e.g. promote, trigger, prevent or impair) the growth or multiplication of a cell (the "target cell") by acting as signalling moieties in conjunction with (e.g. by binding to) cognate cellular receptors. Such polypeptides may be referred to herein as bacterial cytokines.

The RP-factors of the invention therefore inlcude bacterial cytokines which may or may not be capable of resuscitating dormant, moribund or latent cells (e.g. dormant bacterial cells) and/or exhibit growth-stimulatory activity with respect to growing cells (e.g. growing bacterial cells). They may or may not also be competent to reduce the lag time associated with the growth of cell (e.g. bacterial cell) cultures. Moreover, some bacterial cytokines which fall within the scope of the term "RP-factor" as defined herein may even prevent or impair the growth of the target cells (particularly where the target cells are eukaryotic (e.g. mammalian) cells).

The RP-factors of the invention may fall into at least two functional classes: autosignalling factors and allosignalling factors. Autosignalling factors act to regulate the growth of the bacterial cell in which they were expressed (i.e. they act as bacterial autocrine factors), while allosignallers act to regulate the growth of other cells (i.e. they act as bacterial paracrine factors). Autosignalling factors therefore act as self-regulators of bacterial cell growth, and may be essential for viability and/or growth. Some RP-factors may function as both auto- and allosignalling cytokines.

Allosignalling factors may exhibit a range of different specificities. Some may act solely on other bacterial cells of the same species as the cell in which they were expressed ("homoactive" factors), while others may act on cells of one or more other species ("heteroactive" factors). Heteroactive factors may exhibit a broad range of specificity: they may act on several different species (for example, in a genus-specific manner), or may be species-specific. Some heteroactive bacterial factors may act on eukaryotic cells, and may be specific for particular cell-types. For example, some heteroactive bacterial cytokines (particularly those produced by certain pathogens) may act on mammalian cells (e.g. mammalian epithelial, endothelial or immune cells), and may be tissue- or cell-type specific.

Notwithstanding the above explanation, it is postulated that the specificity of at least some RP-factors may be concentration dependent. In these cases, the specificity of any given RP-factor falls within a continuum, so that an autosignalling RP-factor may mediate cross-talk and so exhibit allosignalling activity when present at sufficiently high concentrations. Similarly, allosignalling RP-factors may exhibit homo- or heteroactivity depending on concentration.

The RP-factor may be translocated through the cell membrane, whereafter it may be secreted into the surrounding environment or remain associated with the surface of the cell. Thus, at least two classes of RP-factor may exist: secreted and non-secreted. The secreted RP-factors are characterised by the presence of a secretory signal sequence (the presence of which is readily recognised by those skilled in the art on the basis of the

presence of DNA and/or amino acid sequence motifs). The non-secreted RP-factors may be cell-associated or cytosolic factors. Both classes of RP-factor may exist in a single cellular source (e.g. in a single bacterial source). Both classes of RP-factor find application in the invention.

Non-secreted RP-factors may act in at least four different ways: (a) as a membrane-anchored juxtacrine factor mediating a growth regulating signal between two different cells in close physical proximity or contact; and/or (b) as an intercellular signalling moiety upon cleavage by an enzyme (e.g. a convertase, as herein defined) which releases a soluble signalling moiety into the extracellular milieu; and/or (c) as an autocrine factor *via* binding to cognate receptors located on the surface of the cell in which the non-secreted factor is expressed or acting entirely intracellularly; and/or (d) as a cognate receptor for another non-secreted or secreted RP-factor.

Thus, the RP-factors of the invention may include the nine factors identified by the sequences shown in Fig. 1A and the five factors identified by those shown in Fig 1B, together with their species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms (*vide infra*).

Preferably, the RP-factors of the invention are species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms of any one of the nine factors identified by the sequences shown in Fig. 1A and the five factors identified by those shown in Fig. 1B.

The RP-factors may be synthesised in the form of a precursor which is processed to produce a mature form. Such processing may proceed *via* various intermediate (pro-) forms. Such precursors, intermediate forms and mature proteins are all intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise. As used herein, the term "pro-RP-factor" specifically defines any of various precursors (which may or may not be active) of a mature RP-factor.

The processing may comprise proteolytic cleavage and/or secretion. The precursors may be inactive, and become active on processing as a mature form. The precursors may comprise proteins having secretory leader sequences which are removed during secretion (pre- forms). Such forms are herein referred to as "pre-RP-factor or pre-pro-RP-factors". As explained above, such pre- or prepro- forms are also intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise.

Processing may be attendant on the binding of an RP-factor precursor to a cognate receptor. Such receptors may then directly (or indirectly) cleave the precursor to produce a more mature form of the RP-factor. Such processing may occur as a cascade, involving several receptor-processing complexes, and so ultimately result in the production of a mature RP-factor which then acts as a signalling moiety by binding to a terminal (signal transducing) receptor.

In such processing, the proximal (or intermediate) receptors may function as convertases, and the terminal receptor as a signal transducer. However, a receptor may function as both a convertase and a signal transducer. As used herein, the term "convertase" is intended to define a molecule which binds an RP-factor precursor and (directly or indirectly) processes it to produce a more mature form. They may, for example, have protease activity.

The receptors/convertases discussed above may be disposed at the cell surface (e.g. membrane bound), cytosolic or extracellular.

Preferably, the RP-factor is derived from a bacterium (e.g. a pathogenic bacterium). Particularly preferred are RP-factors derived from high G+C Gram-positive bacteria. However, the inventors have also discovered RP-factor family members in representatives of the low G+C Gram-positive organisms, including *Bacillus subtilis* and clostridia. Thus, RP-factors derived from low G+C Gram-positive bacteria (e.g. pathogenic low G+C Gram-positive bacteria) are also preferred according to the

invention. Examples of the latter include: *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp. and *Lactobacillus* spp..

The invention also contemplates homologues, allelic forms, species variants, derivatives, muteins or equivalents of the RP-factors and RP-factor receptors/convertases of the invention.

Preferably, the homologues, derivatives, muteins or equivalents of the RP-factor of the invention have at least 20% identity with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% identity, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

The homologues, derivatives, muteins or equivalents of the RP-factor of the invention may have at least 25% homology with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% homology with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

The invention also contemplates chimaeric RP-factors. These are factors which comprise one or more heterologous domains. In this context, a heterologous domain is a portion of an RP-factor which is derived from a different RP-factor to that from which the other domain(s) with which it is associated are derived. Such chimaeric RP-factors find particular utility in applications where the specificity and/or activity of the RP-factor is manipulated or altered.

The invention also contemplates all individual functional domains of the RP-factors of the invention as separate and independent entities.

The invention also contemplates recombinant RP-factor. As used herein, the term "recombinant" is intended to define material which has been produced by that body of techniques collectively known as "recombinant DNA technology" (for example, using the nucleic acid, vectors and or host cells described *infra*).

Cognate receptors

In some cases, the cognate cellular receptor is a cell surface receptor: in other cases, it is a cytosolic receptor with which the cytokine interacts after uptake by the target cell. The receptors with which the RP-factors and/or bacterial cytokines of the invention interact may share certain structural motifs with the RP-factors/cytokines themselves. In particular, the receptors may contain a ligand binding domain which is structurally similar to the signalling domain of the cognate RP-factor/cytokine.

The receptors may also comprise a membrane anchor domain and a wall spanning domain.

Preferably, the cognate receptor comprises a receptor domain as hereinbelow defined and/or a wall spanning domain domain as hereinbelow defined and/or a membrane anchor.

Particularly preferred are cognate receptors comprising the amino acid sequence of MtubZ94752 as shown in Fig. 1A or the amino acid sequence of YabE from *B. subtilis* as shown in Fig. 1B.

The cognate receptors may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added,

deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The cognate receptors may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with the amino acid sequence of MtubZ94752 as shown in Fig. 1A or the amino acid sequence of YabE from *B. subtilis* as shown in Fig. 1B, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

RP-factor/cognate receptor domain structure

The RP-factors of the invention (including the bacterial cytokines as also defined herein) and their cognate receptors may comprise a plurality of discrete domains. These domains may be functionally and/or structurally distinct.

The RP-factors of the invention may be characterised by the presence of at least two functional domains: a secretory signal sequence (which may be wholly or partially absent in the active form of the factor) and a signalling domain. The signalling domain may fall into one of at least two distinct classes described in more detail *infra*.

Many RP-factors also comprise a third functional domain which mediates a physical association with the surface of the target cell (hereinafter referred to as the "localizing domain" and described in more detail *infra*).

The RP-factors of the invention may further comprise a specificity-determining domain, which may function in conjunction with the signalling domain.

Non-secreted RP-factors may further comprise a wall-spanning domain (described in more detail *infra*) and/or a membrane anchor.

The gross structure and/or amino acid sequence of the aforementioned domains may vary considerably. In particular, the structure of the surface localizing domain may differ according to the structure of the cell-wall of the target cell. For example, the surface localizing domain may fall into one of at least two distinct classes: class I (which may act on peptidoglycan) and class II (which may act on the outer lipid envelope found in mycobacteria).

The cognate receptors of the invention may be characterised by the presence of at least two functional domains: a receptor domain and a wall spanning domain. They may also comprise a membrane anchor. The receptor domain may be structurally similar to the signalling domain of the cognate RP-factor (as described in more detail *infra*).

Receptor/signalling domain, class I

This domain may be associated with RP-factors from high G+C Gram-positive bacteria (such as mycobacteria and *Micrococcus* spp.) and/or their cognate receptors. When present on RP-factors, the domain may be involved in receptor binding, and may for example bind a structurally similar domain on a cognate receptor. Thus, when present as part of an RP-factor of the invention, the domain is termed the "signalling domain", and when present in the cognate receptor, the domain is termed the "receptor domain".

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks in any one of the 9 sequences set out in Figure 1A.

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks and dots in any one of the 9 sequences set out in Figure 1A.

In particularly preferred embodiments, the domain may comprise a sequence of amino

acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 9 sequences set out in Figure 1A.

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case type in any one of the 9 sequences set out in Figure 1A.

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Receptor/signalling domain, class II

This domain may be associated with RP-factors from low G+C Gram-positive bacteria (such as bacilli and clostridia) and/or their cognate receptors. When present on RP-factors, the domain may be involved in receptor binding, and may for example bind a structurally similar domain on a cognate receptor. Thus, when present as part of an RP-factor of the invention, the domain is termed the "signalling domain", and when present in the cognate receptor, the domain is termed the "receptor domain".

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks in any one of the 5 sequences set out in Figure 1B(B).

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks and dots in any one of the 5 sequences set out in Figure 1B(B).

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 5 sequences set out in Figure 1B(B).

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case type in any one of the 5 sequences set out in Figure 1B(B).

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Wall spanning domain

This domain may be associated with non-secreted RP-factors (e.g. cell-associated RP-factors or RP-factors which act as juxtacrine factors) and with the cognate receptors of the RP-factors of the invention. When present, the domain is involved in mediating an interaction with the cell wall such that the RP-factor/receptor as a whole may span it.

The wall spanning domain may therefore be bounded by cytosolic and extracellular regions *in vivo*. The domain is often associated with a membrane anchor, the two structural elements acting in concert to maintain the RP-factor/receptor at the cell surface.

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case and indexed by hashes (#) in any one of the 5 sequences set out in Figure 1B(A).

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case and indexed by hashes and dots in any one of the 5 sequences set out in Figure 1B(A).

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 5 sequences set out in Figure 1B(A).

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case type in any one of the 5 sequences set out in Figure 1B(A).

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Localizing domain, class I

This domain may be present in secreted RP-factors, and may mediate a physical association with the surface of the target cell by acting to bind peptidoglycan or some other surface component(s). It may therefore act to increase the local concentration of the cytokine at the target cell surface, so promoting activity by increasing the local concentration of RP-factor in the immediate vicinity of the cognate receptor. Localizing domains may therefore be a characteristic feature of allosignalling bacterial cytokines, and may be absent in autosignalling factors or *vice versa*. For example, when present in autosignalling factors, localizing domains may act to retain the factor at or near the cell surface after secretion through the cell membrane.

When present, the localizing domain may confer important binding properties on the RP-factor, whereby binding to cognate receptor is biphasic and characterised by a primary (relatively unspecific) association with the cell surface followed by a secondary (relatively highly specific) association with the cognate receptor.

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks in any one of the 10 sequences set out in Figure 1C.

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks and dots in any one of the 10 sequences set out in Figure 1C.

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 10 sequences set out in Figure 1C.

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues set out in any one of the 10 sequences set out in Figure 1C.

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Localizing domain, class II

This domain may be present in secreted RP-factors, and may mediate a physical association with the surface of the target cell by acting to bind the outer lipid envelope present in mycobacteria. It may therefore act to increase the local concentration of the cytokine at the target cell surface, so promoting activity by increasing the local concentration of RP-factor in the immediate vicinity of the cognate receptor. Localizing domains may therefore be a characteristic feature of allosignalling bacterial cytokines, and may be absent in autosignalling factors.

When present, the localizing domain may confer important binding properties on the RP-factor, whereby binding to cognate receptor is biphasic and characterised by a

primary (relatively unspecific) association with the cell surface followed by a secondary (relatively highly specific) association with the cognate receptor.

The domain may comprise an alanine plus proline-rich segment, such as one or more of the amino acid motifs 'A', A, B, B', C, 'C, D, D* and D' (any one of which may be tandemly repeated) as set out in Figure 1D.

In preferred embodiments, the domain may comprise a sequence of amino acid residues corresponding to residues 158-322 of MtubMTV043 as shown in Figure 1D or to that of residues 45-112 of MtubMTV008 as shown in Figure 1A.

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

The term "isolated" is used herein to indicate that the factor exists in a physical milieu distinct from that in which it occurs in nature. For example, the isolated factor may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs. The absolute level of purity is not critical, and those skilled in the art can readily determine appropriate levels of purity according to the use to which the factor is to be put.

In many circumstances, the isolated factor will form part of a composition (for example a more or less crude extract containing many other proteins and substances), buffer

system or pharmaceutical excipient, which may for example contain other components (including other proteins, such as albumin).

In other circumstances, the isolated protein may be purified to essential homogeneity, for example as determined by PAGE or column chromatography (for example HPLC or mass spectrometry). In preferred embodiments, the isolated RP-factor of the invention is essentially the sole active RP-factor in a given composition. Particularly preferred are compositions in which an RP-factor (or a particular species, homologue, mutein, derivative or equivalent thereof) is present as the sole active ingredient in a pharmaceutical composition.

The RP-factor for use in the invention need not be isolated in the sense defined above, however. For example, more or less crude culture supernatants (e.g. "spent" medium) may contain sufficient concentrations of RP-factor for use in several applications. Preferably, such supernatants are fractionated and/or extracted (see below), but in many circumstances they may be used without pretreatment. They are preferably derived from spent media used to culture RP-factor-producing microorganisms (for example, the bacterial sources described *infra*). The supernatants are preferably sterile. They may be treated in various ways, for example by concentration, filtration, centrifugation, spray drying, dialysis and/or lyophilisation. Conveniently, the culture supernatants are simply centrifuged to remove cells/cell debris and filtered.

Such supernatants find utility in diagnostic kits and methods, for example in the diagnostic kits and methods described *infra*. They also find utility in the recovery from various samples of culturable microorganisms (e.g. from soil, food, marine, freshwater, or tissue samples) or from samples taken from an organism (e.g. a human or animal).

The culture supernatants may also be used as supplements in various culturing substrates, for example in culture or transport media. The culture medium may take any convenient form, such as for example agar plates, broths, slopes, coated dipsticks, coated probes, membranes, coated or filled wells or films. The medium may be a defined or

complex medium, and may contain indicator dyes to facilitate identification of cultured microorganisms. Preferably, the medium is suitable for the culturing or transport of bacteria, for example *Mycobacterium* spp..

The term "isolated" as applied to the other materials of the invention (for example, the genes and other nucleic acids encoding the RP-factor and their cognate receptors/convertases) is to be interpreted mutatis mutandis. Thus, as applied to nucleic acid (e.g. RNA or DNA or (structural) genes), the isolated nucleic acid may be present in any of a wide variety of vectors and in any of a wide variety of host cells (or other milieu, such as buffers, viruses or cellular extracts).

The term "family", as applied to the proteins of the invention, is used herein to indicate a group of proteins which share substantial sequence similarities, either at the level of the primary sequence of the proteins themselves, or at the level of the DNA encoding them. The sequence similarities may extend over the entire protein/gene, or may be limited to particular regions or domains. Similarities may be based on nucleotide/amino acid sequence identity as well as similarity (for example, those skilled in the art recognise certain amino acids as similar, and identify differences based on switches of similar amino acids as conservative changes). Some members of a protein family may be related in the sense that they share a common evolutionary ancestry, and such related proteins may herein be referred to as homologues. The members of a protein family do not necessarily share the same biochemical properties or biological functions, though their similarities are usually reflected in common functional features (such as effector binding sites and substrates).

The criteria by which protein families are recognised are well-known in the art, and include computer analysis of large collections of sequences at the level of DNA and protein as well as biochemical techniques such as hybridisation analysis and enzymatic assays (see for example Pearson and Lipman (1988), PNAS USA, 85: 2444).

Thus, the RP-factors of the invention include the factors shown in Fig. 1A and 1B, together with their species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms (*vide infra*). Preferably, the RP-factors of the invention are species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms of any one of the proteins represented in Fig. 1A and Fig. 1B.

The RP-factors may be synthesised in the form of a precursor which is processed to produce a mature form. Such processing may proceed *via* various intermediate (pro-) forms. Such precursors, intermediate forms and mature proteins are all intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise. As used herein, the term "pro-RP-factor" specifically defines any of various precursors (which may or may not be active) of a mature RP-factor.

The processing may comprise proteolytic cleavage and/or secretion. The precursors may be inactive, and become active on processing as a mature form. The precursors may comprise proteins having secretory leader sequences which are removed during secretion (pre- forms). Such forms are herein referred to as "pre-RP-factor or pre-pro-RP-factors". As explained above, such pre- or prepro- forms are also intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise.

Processing may be attendant on the binding of an RP-factor precursor to a cognate receptor. Such receptors may then directly (or indirectly) cleave the precursor to produce a more mature form of the RP-factor. Such processing may occur as a cascade, involving several receptor-processing complexes, and so ultimately result in the production of a mature RP-factor which then acts as a signalling moiety by binding to a terminal (signal transducing) receptor.

In such processing, the proximal (or intermediate) receptors may function as convertases, and the terminal receptor as a signal transducer. However, a receptor may function as both a convertase and a signal transducer. As used herein, the term

"convertase" is intended to define a molecule which binds an RP-factor precursor and (directly or indirectly) processes it to produce a more mature form. They may, for example, have protease activity.

The receptors/convertases discussed above may be disposed at the cell surface (e.g. membrane bound), cytosolic or extracellular.

Preferably, the RP-factor is derived from a bacterium (e.g. a pathogenic bacterium). Particularly preferred are RP-factors derived from high G+C Gram-positive bacteria.

The term "derived from" as applied to a defined source is intended to define not only a source in the sense of it being the *physical* origin for the material, but also to define material which has structural and/or functional characteristics which correspond to those of material which does originate from the reference source. Thus, a protein "derived from" a given source need not necessarily have been purified from that source.

The term "high G+C Gram-positive bacterium" is a term of art defining a particular class of evolutionarily related bacteria. The class includes *Micrococcus* spp. (e.g. *M. luteus*), *Mycobacterium* spp. (for example a fast- or slow-growing mycobacterium, e.g. *M. tuberculosis*, *M. leprae*, *M. smegmatis* or *M. bovis*), *Streptomyces* spp. (e.g. *S. rimosus* and *S. coelicolor*) and *Corynebacterium* spp. (e.g. *C. glutamicum*). Preferred according to the invention are RP-factors/cognate receptors/convertases derived from mycobacteria ("mycobacterial RP-factors/RP-factor receptors/convertases").

The invention also contemplates homologues, allelic forms, species variants, derivatives, muteins or equivalents of the RP-factors and RP-factor receptors/convertases of the invention.

The term "homologue" is used herein in two distinct senses. It is used *sensu stricto* to define the corresponding protein from a different organism (i.e. a species variant), in which case there is a direct evolutionary relationship between the protein and its

homologue. This may be reflected in a structural and functional equivalence, the protein and its homologue performing the same role in each organism.

The term is also used herein *sensu lato* to define a protein which is structurally *similar* (i.e. not necessarily related and/or structurally and functionally equivalent) to a given (reference) RP-factor. In this sense, homology is recognised on the basis of purely structural criteria by the presence of amino acid sequence identities and/or conservative amino acid changes (as set out by Dayhoff *et alia*, *Atlas of protein structure* vol. 5, National BioMed Fd'n, Washington D.C., 1979).

For the purposes of the invention, homologues may be recognised as those proteins the corresponding DNAs of which are capable of specifically or selectively cross-hybridising, or which can cross-hybridise under selective, appropriate and/or appropriately stringent hybridisation conditions.

The term "selectively or specifically (cross)hybridisable" in this context indicates that the sequences of the corresponding ssDNAs are such that binding to a unique (or small class) of homologous sequences can be obtained under more or less stringent hybridisation conditions. This method of the invention is not dependent on any particular hybridisation conditions, which can readily be determined by the skilled worker (e.g. by routine trial and error or on the basis of thermodynamic considerations).

Preferably, the homologues, derivatives, muteins or equivalents of the RP-factor of the invention have at least 20% identity with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% identity, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

The homologues, derivatives, muteins or equivalents of the RP-factor of the invention may have at least 25% homology with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% homology with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

The term "derivative" as applied herein to the proteins (e.g. the RP-factors or RP-factor receptors/convertases) of the invention is used to define proteins which are modified versions of the proteins of the invention. Such derivatives may include fusion proteins, in which the proteins of the invention have been fused to one or more different proteins or peptides (for example an antibody or a protein domain conferring a biochemical activity, to act as a label, or to facilitate purification).

The derivatives may also be products of synthetic processes which use a protein of the invention as a starting material or reactant.

The term "mutein" is used herein to define proteins that are mutant forms of the proteins of the invention, i.e. proteins in which one or more amino acids have been added, deleted or substituted. The muteins of the invention therefore include fragments, truncates and fusion proteins (e.g. comprising fused immunoglobulin, receptor, convertase or enzyme moieties).

The muteins of the invention also include proteins in which mutations have been introduced which effectively promote or impair one or more activities of the protein, for example mutations which promote or impair the function of a receptor, a recognition sequence or an effector binding site.

Muteins may be produced by any convenient method. Conveniently, site-directed mutagenesis with mutagenic oligonucleotides may be employed using a double stranded template (pBluescript KS II construct containing the RP-factor or RP-factor receptor/convertase gene), (e.g. Chameleon™ or QuikChange™ - Stratagene™). After verifying each mutant derivative by sequencing, the mutated gene is excised and inserted into a suitable vector so that the modified protein can be over-expressed and purified.

Preferred mutant forms are truncates consisting (or consisting essentially) of the RP-factor signalling domain or the RP-factor specificity-determining factor, or of the ligand binding domain of the RP-factor receptor, or combinations of two or more of the foregoing.

The invention also contemplates chimaeric RP-factors. These are factors which comprise one or more heterologous domains. In this context, a heterologous domain is a portion of an RP-factor which is derived from a different RP-factor to that from which the other domain(s) with which it is associated are derived. Such chimaeric RP-factors find particular utility in applications where the specificity and/or activity of the RP-factor is manipulated or altered.

Useful in the construction of such chimaeric RP-factors are DNA fragments or cassettes consisting essentially of DNA encoding selected domains (for example, the signalling domain or the specificity-determining domain), the fragment or cassette optionally being bounded by one or more restriction endonuclease cleavage sites or cloning sites. The invention also contemplates concatenated domain cassettes, as well as mutant RP-factor structural genes which have cloning sites (e.g. one or more restriction endonuclease cleavage sites) located in one or more interdomain regions.

The term equivalent as used herein and applied to the materials of the invention defines materials (e.g. proteins, DNA etc.) which exhibit substantially the same functions as those of the materials of the invention while differing in structure (e.g. nucleotide or amino acid sequence). Such equivalents may be generated for example by identifying

sequences of functional importance (e.g. by identifying conserved or canonical sequences or by mutagenesis followed by functional assay), selecting an amino acid sequence on that basis and then synthesising a peptide based on the selected amino acid sequence. Such synthesis can be achieved by any of many different methods known in the art, including solid phase peptide synthesis (to generate synthetic peptides) and the assembly (and subsequent cloning) of oligonucleotides.

The homologues, fragments, muteins, equivalents or derivatives of the proteins of the invention may also be defined *inter alia* as those proteins which cross-react with antibodies to the proteins of the invention, and in particular which cross-react with antibodies directed against any of the specific proteins shown Fig. 1A or Fig. 1B.

The invention also contemplates all individual functional domains of the RP-factors of the invention as separate and independent entities.

The invention also contemplates recombinant RP-factor. As used herein, the term "recombinant" is intended to define material which has been produced by that body of techniques collectively known as "recombinant DNA technology" (for example, using the nucleic acid, vectors and or host cells described *infra*).

The invention also contemplates a pharmaceutical composition (e.g. a vaccine) comprising the RP-factor or RP-factor receptor/convertase (or homologue, species variant, allelic form, derivative, mutein or equivalent thereof) of the invention.

A pharmaceutical composition is a solid or liquid composition in a form, concentration and level of purity suitable for administration to a patient (e.g. a human or animal patient) upon which administration it can elicit the desired physiological changes. The vaccines of the invention may include any suitable adjuvant (e.g. Freund's adjuvant, BCG or BCG extracts).

In another aspect, the invention relates to a pharmaceutical composition comprising the material of the invention which is: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

In another aspect, the invention relates to an antibody (or antibody derivative) specific for the RP-factor (or homologue, derivative, mutein or equivalent thereof) of the invention.

The antibody is preferably in a form suitable for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or formulated in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration. The antibody may be labelled and/or immortalised and/or conjugated to another moiety, and such embodiments find particular utility in diagnostic applications.

According to another aspect of the invention there is provided an isolated or recombinant RP-factor receptor.

The receptor/convertase may be derived from any of the sources hereinbefore described, for example from a bacterial source (e.g. a pathogenic bacterial source). Such sources include high G+C Gram-positives, *Micrococcus* spp. (e.g. *M. luteus*); or *Mycobacterium* spp. (for example a fast- or slow-growing mycobacterium, e.g. *M. tuberculosis*, *M. leprae*, *M. smegmatis* or *M. bovis*); or *Streptomyces* spp. (e.g. *S. rimosus* and *S. coelicolor*); or *Corynebacterium* spp. (e.g. *C. glutamicum*).

The invention also contemplates homologues, derivatives, muteins or equivalents of the receptors/convertases of the invention, as well as recombinant RP-factor receptors/convertases (as hereinbefore defined).

The invention also contemplates a pharmaceutical composition (e.g. a vaccine) comprising the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) of the invention.

Preferably, the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) or pharmaceutical composition is: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated is an antibody (or antibody derivative) specific for the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) of the invention. The antibody may be: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated is an RP-factor antagonist or inhibitor.

Preferably, the antagonist or inhibitor comprises: (a) the antibody of the invention; and/or (b) the receptor/convertase of the invention; and/or (c) an RP-factor mutein comprising an RP-factor specificity-determining domain, which for example lacks a functional signalling domain. The receptor may function as an antagonist or inhibitor if administered in soluble form, where it may act as a sink for soluble RP-factor. Preferably, modified receptors consisting of the receptor domain (and lacking the membrane anchor and wall spanning domain) are used as inhibitors or antagonists. Such derivatives may exhibit higher solubility.

The antagonist or inhibitor of the invention is preferably: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated by the invention is an RP-factor agonist, activator or mimetic. Preferably, the agonist, activator or mimetic comprises: (a) the RP-factor receptor/convertase antibody as herein described; and/or (b) an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; and/or (c) an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or (d) operably coupled combinations of any of (a)-(c).

The agonist, activator or mimetic may be: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) formulated in a pharmaceutical excipient, a unit dosage form, in a form suitable for local or systemic administration or in admixture with an antibiotic.

Preferably, the agonist, activator or mimetic may be for use in adjunctive therapy (for example formulated or presented in combination with an antimicrobial agent, e.g. an antibiotic).

The invention also contemplates isolated nucleic acid encoding the RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) or RP-factor receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) of the invention. The nucleic acids of the invention therefore embrace DNA having any sequence so long as it encodes the proteins of the invention. It will be appreciated by those skilled in the art that as a result of degeneracy in the genetic code, any particular amino acid sequence of the invention may be encoded by many different sequences. Thus, the nucleic acid sequence may be selected or optimised, e.g. with respect to the codon usage in any particular host cell.

The invention also contemplates vectors (e.g. an expression vector) comprising the nucleic acid of the invention. The nature of the vector is not critical to the invention. Any suitable vector may be used, including plasmid, virus, bacteriophage, transposon, minichromosome, liposome or mechanical carrier.

The expression vectors of the invention are DNA constructs suitable for expressing DNA which encodes the desired protein product (e.g. RP-factor or RP-factor receptor) which may include: (a) a regulatory element (e.g. a promoter, operator, activator, repressor and/or enhancer), (b) a structural or coding sequence which is transcribed into mRNA and (c) appropriate transcription, translation, initiation and termination sequences. They may also contain sequence encoding any of various tags (e.g. to facilitate subsequent purification of the expressed protein, such as affinity (e.g. His) tags).

Particularly preferred are vectors which comprise an expression element or elements operably linked to the DNA of the invention to provide for expression thereof at suitable levels. Any of a wide variety of expression elements may be used, and the expression element or elements may for example be selected from promoters, enhancers, ribosome binding sites, operators and activating sequences. Such expression elements may comprise an enhancer, and for example may be regulatable, for example being inducible (*via* the addition of an inducer).

As used herein, the term "operably linked" refers to a condition in which portions of a linear DNA sequence are capable of influencing the activity of other portions of the same linear DNA sequence. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The vector may further comprise a positive selectable marker and/or a negative selectable marker. The use of a positive selectable marker facilitates the selection and/or identification of cells containing the vector.

Also contemplated by the invention are host cells comprising the vector of the invention. Any suitable host cell may be used, including prokaryotic host cells (such as *Escherichia*

coli, *Streptomyces* spp. and *Bacillus subtilis*) and eukaryotic host cells.

In another aspect, the invention provides a culture or transport medium comprising the RP-factor (or homologue, derivative, mutein or equivalent thereof) of the invention. The culture medium may take any convenient form, such as for example agar plates, broths, slopes, coated dipsticks, coated probes, membranes, coated or filled wells or films. The medium may be a defined or complex medium, and may contain indicator dyes to facilitate identification of cultured microorganisms. Preferably, the medium is suitable for the culturing or transport of bacteria, for example *Mycobacterium* spp. *Streptomyces* spp. and *Corynebacterium* spp.

The invention also contemplates a nucleic acid probe comprising nucleic acid complementary to the nucleic acids of the invention. Such probes are preferably selectively hybridisable with nucleic acid encoding the proteins (e.g. the RP-factors of RP-factor receptors/convertases) of the invention. They are conveniently single stranded DNA or RNA probes.

The invention also contemplates a diagnostic kit comprising the factor (or homologue, derivative, mutein or equivalent thereof), receptor, antibody, probe or culture medium of the invention.

In another aspect, the invention contemplates antisense DNA corresponding to the nucleic acid encoding the RP-factor or RP-factor receptor/convertase of the invention.

The invention also contemplates a process for producing an antimicrobial drug comprising the steps of: (a) providing an RP-factor receptor; (b) providing candidate drugs; (c) screening the candidate drugs by contacting the RP-factor receptor/convertase with one of the candidate drugs and determining the affinity of the candidate drug for the RP-factor receptor, wherein the affinity is an index of antimicrobial activity, and optionally (d) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (c).

Preferably, the process for producing an antimicrobial drug comprises the steps of: (a) providing an RP-factor receptor/convertase; (b) providing a candidate drug; (c) providing an RP-factor; (d) screening the candidate drugs by contacting the RP-factor receptor/convertase with one of the candidate drugs in the presence of the RP-factor, and then determining the ability of the candidate drug to compete non-productively with the RP-factor for binding to the RP-factor receptor, wherein the competitive binding ability is an index of antimicrobial activity, and optionally (e) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (d).

The invention also covers an antimicrobial drug produced by (or obtainable by) the processes of the invention, and also derivatives thereof.

Also contemplated by the invention is a method for determining the microbiological quality of a product (e.g. a foodstuff, pharmaceutical preparation or medical product) comprising the step of contacting a sample of the product with an RP-factor (for example, an RP-factor as hereinbefore defined). In such methods, the RP-factor preferably forms part of a nutrient composition (e.g. a plate, broth, film or dipstick).

In another aspect, the invention relates to a method of culturing bacterial (e.g. mycobacterial) cells, comprising the step of incubating the cells in a culture medium containing an RP-factor (for example, an RP-factor as hereinbefore defined).

Also contemplated by the invention is an *ex vivo* method of diagnosis, comprising the step of contacting a biological sample with an RP-factor (for example, an RP-factor as hereinbefore defined).

The diagnostic method of the invention preferably includes the step of incubating the culture or transport medium of the invention to permit growth of cells in the biological sample (e.g. bacterial cells).

Also contemplated by the invention is a method of: (a) stimulating the growth of a microorganism; and/or (b) resuscitating a dormant, moribund or latent microorganism; comprising the step of contacting the microorganism with an RP-factor (for example, an RP-factor as hereinbefore defined).

The invention also contemplates a process for producing the recombinant RP-factor or RP-factor receptor/convertase of the invention comprising the steps of: (a) culturing the host cell of the invention, and (b) purifying the factor or receptor/convertase from the cultured host cells (e.g. from a culture supernatant or cell fraction).

Also contemplated by the invention is a process for producing the recombinant RP-factor or receptor/convertase of the invention comprising the steps of: (a) probing a gene library with a nucleic acid probe which is selectively hybridisable with the cognate structural gene to produce a signal which identifies a gene that selectively hybridises to the probe; (b) expressing the gene identified in step (a) (for example by cloning into a host cell, e.g. according to the process as hereinbefore defined) to produce the factor or receptor.

Also covered is a recombinant RP-factor or receptor/convertase obtainable by the above-described process.

Medical applications

The invention permits the isolation, synthesis and rational design of a wide range of novel medicaments and pharmaceuticals for use in therapy, prophylaxis and diagnosis.

The various forms of therapy, prophylaxis and diagnosis in which the materials of the invention find application may involve changing, breaking or perturbing the resuscitation (RP-factor) signal transduction pathway of one or more infecting pathogens.

Thus, the materials of the invention find general application as antimicrobial agents, for example as antibacterial agents. They may therefore be used in the treatment, prophylaxis or diagnosis of microbial (e.g. bacterial) infections, particularly those infections associated with latency (e.g. mycobacterial infections).

Thus, the invention may for example be used to prevent, reduce or interfere with: (a) the resuscitation of a latent (or dormant) pathogen, and/or (b) the growth of a pathogen, and/or (c) the multiplication and spread of a pathogen; and/or (d) the activation of a latent infection (for example a latent bacterial (e.g. mycobacterial) infection).

In general, the materials of the invention may be used to treat conditions in which changing, breaking or perturbing the resuscitation (RP-factor) signal transduction pathway or blockading the RP-factor receptor/convertase associated with an infecting pathogen is indicated.

Particularly useful materials for use in such therapies/prophylactic methods include RP-factor antagonists or inhibitors. Such antagonists or inhibitors may comprise antibodies to the RP-factor or to the RP-factor receptor/convertase as herein defined; the RP-factor receptor/convertase of the invention; an RP-factor mutein, e.g. which comprises an altered RP-factor specificity-determining domain and/or which lacks a functional signalling domain.

RP-factor antibodies act to sequester and ultimately eliminate endogenous RP-factors in a patient bearing a latent microbial infection.

RP-factor receptor antibodies bind non-productively to the receptors associated with the infecting pathogen. Antibodies to the convertase inactivate (e.g. by steric inhibition) the convertase activity and so prevent maturation of the RP-factor. The antibodies may therefore competitively inhibit the binding of endogenous RP-factor to the receptors/convertases associated with the infecting pathogen. Alternatively, they may

bind with high affinity (and/or essentially irreversibly) to the RP-factor receptors/convertases and so block RP-factor-ligand binding or RP-factor maturation. A similar activity is displayed by the RP-factor muteins having altered specificity and/or signalling activity.

In either case, the RP-factor-RP-receptor/convertase binding required for resuscitation of latent pathogens, growth of the pathogen and/or progression of the disease state is perturbed, reduced or abolished.

RP-factor receptors for use as therapeutics in such methods are uncoupled from the signal transduction pathway with which they are normally associated. Thus, they are preferably free (i.e. in soluble or dispersible) form and/or not membrane bound. In this way, effective circulating or systemic concentrations of the free RP-factor receptor can be established and maintained in a patient. In this form, the RP-factor receptors act as RP-factor sinks, and titrate out (and preferably ultimately eliminate) endogenous RP-factors in a patient bearing a latent microbial infection. The receptors therefore reduce or prevent activation of the (latent) pathogen and/or stimulation of pathogen growth, so slowing or halting the progression of the infection.

In another aspect, the invention may be used to resuscitate or assist in resuscitating (or activate or assist in activating) a latent (dormant) pathogenic microbe *in vivo* thereby to potentiate adjunctive antimicrobial therapy. The adjunctive antimicrobial therapies for use in such applications are those which depend for full efficacy on a non-latent or active (e.g. growing or replicating) target pathogen population (for example adjunctive therapies based on certain types of antibiotic). Thus, the materials of the invention may act synergistically with various antimicrobial compounds in antimicrobial therapy.

In a preferred embodiment, the invention is used to potentiate the antimicrobial therapy of tuberculosis, for example involving co-administration of one or more of isoniazid, rifampicin, pyrzinamide and/or ethambutol (or streptomycin).

Particularly useful materials for use in such therapies include for example the RP-factors of the invention, RP-factor agonists, activators and mimetics. Such agonists, activators or mimetics may comprise: the RP-factor receptor antibodies as hereinbefore described; the RP-factor convertase as hereinbefore defined; an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or operably coupled combinations thereof.

The RP-factor receptor antibodies for use in such methods are those which serve to trigger an efferent signal transduction pathway at the RP-factor receptor. They may therefore act as RP-factor mimetics, breaking latency/dormancy and acting to resuscitate the pathogen.

Particularly useful in such methods are mutant RP-factors having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified). Such mutant RP-factors may be active against a broad range of pathogens (e.g. against substantially all pathogenic or infective mycobacteria) or targeted against specific pathogens (for example, *M. tuberculosis* and *M. leprae*).

The antibodies, RP-factors, receptors and convertases discussed above may be administered directly or *via* a live vaccine vehicle. Such live vaccines vehicles comprise microorganisms which have been genetically engineered to express (and preferably secrete) the therapeutically active antibodies, RP-factors, receptors and convertases of the invention *in vivo*.

The invention therefore finds application in the treatment of a wide variety of microbial infections, and finds particular application in the treatment of latent microbial (e.g. bacterial) infections.

In preferred embodiments, the invention finds application in the treatment of actinomycete or mycobacterial infections, for example those involving *M. tuberculosis,*

M. leprae, M. bovis, M. kansasii and *M. avium*.

Other infections which may be treated according to the invention include those involving *Corynebacterium* spp. (including *Corynebacterium diphtheriae*), *Tropheryma whippelii*, *Nocardia* spp. (including *Nocardia asteroides* and *Nocardia brasiliensis*), *Streptomyces* spp. (including *Streptomyces griseus, Streptomyces paraguayensis* and *Streptomyces somaliensis*), *Actinomadura* spp., *Nocardiopsis* spp., *Rhodococcus* spp., *Gordona* spp., *Tsukamurella* spp. and *Oerskovia* spp. as well as other pathogenic organisms from the group referred to as high G+C Gram-positive bacteria. Other infections which may be treated include those involving pathogenic low G+C Gram-positive bacteria (e.g. *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp. and *Lactobacillus* spp.).

The invention may also be embodied in various vaccines or immunotherapeutic agents.

Such vaccines or agents target one or more elements of the RP-factor mediated signal transduction pathway described herein (and in particular, the RP-factor or RP-factor receptors/convertases themselves). Thus, the RP-factors may be administered as part of a vaccine or immunotherapeutic composition to elicit an immune response directed against endogenous RP-factor in the patient, so reducing, preventing activation of the pathogen and so slowing or halting the progression of the infection.

Alternatively (or in addition), the RP-factor receptors/convertases may be administered as part of a vaccine or immunotherapeutic composition to elicit an immune response directed against receptors for pathogen-borne RP-factor in the patient. In this way, cellular and/or humoral immune responses may be stimulated against the pathogen(s) and/or activation of a latent pathogen (or its continued growth or multiplication) *via* the RP-factor signal transduction pathway may be reduced or prevented, so slowing or halting the progression of the infection.

The invention also finds application in the preparation of live vaccines: attenuated

microbial strains can be constructed in which the gene(s) encoding (or regulating the expression or activity of) one or more RP-factors are mutated. Such attenuated vaccines may be based on mutant strains of actinomycetes, mycobacteria (for example *M. tuberculosis, M. leprae, M. bovis* (such as *M. bovis* BCG), *M. kanasii* and *M. avium*), *Corynebacterium* spp. (including *Corynebacterium diphtheriae*), *Tropheryma whippelii, Nocardia* spp. (including *Nocardia asteroides* and *Nocardia brasiliensis*), *Streptomyces* spp. (including *Streptomyces griseus, Streptomyces paraguayensis* and *Streptomyces somaliensis*), *Actinomadura* spp., *Nocardiopsis* spp., *Rhodococcus* spp., *Gordona* spp., *Tsukamurella* spp. and *Oerskovia* spp. as well as other pathogenic organisms from the group referred to as high G+C Gram-positive bacteria.

Particularly useful in such attenuated vaccines are strains bearing mutated RP-factor-encoding genes. Such mutations may be frameshift, deletion, insertion and/or substitution mutations. In preferred embodiments the mutations are null mutations (e.g. non-reverting null mutations), and may prevent growth of the microbe (i.e. "attenuate" it). In other embodiments the mutations may result in the expression of mutant RP-factors having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified) and/or which lack a functional signalling domain. Such mutant RP-factors may bind with high affinity (and/or essentially irreversibly) and non-productively to the RP-factor receptors/convertases and so block RP-factor-ligand binding or RP-factor maturation. The attenuated microbial strains of the invention may also bear mutations in other genes (for example, in other genes essential to growth), and may also bear one or more genetic marker elements.

Biotechnological applications

It is widely recognised that the great majority (probably well in excess of 99%) of soil organisms have not yet been cultured. Hitherto uncultured organisms are also expected to exist in other sources. The present invention may be used to permit the recovery of such organisms by culture from any source. Thus, the invention provides a way of unlocking an immense reservoir of biodiversity that is known to exist, but is presently

inaccessible.

Thus, the present invention provides an unprecedented resource from which libraries of potentially useful microorganisms and biomolecules can be generated. Such libraries can then be used in screening methods to search for medically or industrially useful products.

Thus, in another aspect the invention provides a process for producing a library of biomolecules comprising the steps of: (a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived); (b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture; and (c) isolating microorganisms from the culture of step (b).

The process may further comprise the step of screening the isolated microorganisms for those which elaborate one or more biomolecules of interest (for example a metabolite, enzyme, antibiotic (e.g. antiviral, antibacterial or antifungal agent) or toxin).

Also contemplated is a biomolecule produced by (or obtainable by) the above process, or a derivative thereof.

In another aspect, the invention provides a process for producing a library of microorganisms (e.g. bacteria) comprising the steps of: (a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived sample); (b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture; (c) isolating microorganisms from the culture of step (b).

Also contemplated is a microbe produced by (or obtainable by) the above process, or a derivative (e.g. mutant) thereof.

Exemplification

The invention will now be described in more detail with reference to several Examples. These are for exemplary purposes only and are not intended to limit the invention in any way.

Explanation of the Figures

Figure 1: Part A. Multiple sequence alignment of the predicted amino-acid sequences of RP-factor-like gene products from *M. luteus*, *M. tuberculosis*, *M. leprae* and *Streptomyces coelicolor*. Proteins similar to the RP-factor are derived from *M. tuberculosis* (accession nos. U38939, nt 2406-2765, and Z81368, nt 33932-34396) and *M. leprae* (accession nos. L01095, nt 12292-12759, and L04666, nt 25446-24921). The DNA sequences of interest in accession Z81368 are also encompassed by accession AD000010. N-terminal residues corresponding to predicted Gram-positive signal sequences are underlined. The *M. leprae* L04666 sequence may also contain a short, 32 aa signal peptide.

Part B. Multiple sequence alignment of gene products related to YabE of *Bacillus subtilis*. The alignment is given in two parts (A and B), with aligned residues in upper case. Those residues which are conserved (or conservatively substituted) in two or more sequences are in bold. In Part A, perfectly conserved residues are marked with a hash (#) and conservative substitutions with a dot (.). Cperfring is an incomplete ORF1 from *Clostridium perfringens* (Acc. No. UO4966); Caceto506 is an incomplete ORF from contig 506, *Clostridium acetobutylicum* genome sequencing project. YocH from *B. subtilis* and YabE from *B. subtilis* are YocH and YabE predicted gene products from the *B. subtilis* genome sequencing project (Acc. Nos. BG13521 and P37456).

Part C. Alignment of the RP-factor C-terminal domain with known and hypothetical wall-associated proteins from other organisms. Perfectly conserved

residues are marked with an asterisk, those conserved in at least 7 sequences are marked with a dot (.).

Part D. Motifs in the C-terminus (residues 158-322) of MtubMTV043.

Part E. Alignment between the predicted amino acid sequence of the *M. luteus* RP-factor and p60 proteins from *Listeria* spp. Many of the residues that are conserved in the alignment between the C-terminal portion of the *M. luteus* RP-factor (residues 125-220) and the *L. monocytogenes* EGD p60 protein (residues 158-245), are also conserved in the p60 protein from six other *Listeria* spp.

Figure 2: Part A. The sequence of the RP-factor-encoding gene and its predicted product. The nucleotide sequence is in lower case with PCR primers in bold. The predicted protein sequence is in upper case bold (single letter code). Protein and peptide microsequence data used for oligonucleotide design are in upper case italics.

Part B. The sequence of a 299 base pair DNA fragment encoding part of an RP-factor from *Streptomyces coelicolor*. The deduced amino acid sequence is given below the DNA sequence using the single letter amino acid code.

Figure 3: The elution profile of the resuscitation activity. Fractions eluted from the DEAE-sepharose column (see Materials and Methods) with 0.25 M KCl were applied to a Mono Q column which was developed with a 20ml linear gradient from 0.08 to 0.28 M KCl in 10 mM Tris-Cl buffer supplemented by 10% glycerol, pH 7.4. 10 ml of a diluted suspension of starved cells (CFU 3.10^6cells.ml^{-1}, total count $1.2.10^9$cells.ml^{-1}) were added to 200 ml of LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract containing of 2 μl of each fraction in 5-10 replicates in the Bioscreen instrument. For details see Materials and Methods. **A**: absorbance at 280nm and magnitude of KCl concentration. **B**: resuscitation activity. **C**: SDS-PAGE profile of the fractions following DEAE-cellulose and Mono Q chromatography. Lanes : 1, markers (94,000, 67,000, 43,000, 30,000, 20,100, 14,400); 2, fraction from DEAE-cellulose column; 3, purifed

preparation (fraction number 8 from the Mono Q -column). **D**: Reduction of apparent lag phase of viable cells. 10 μl of a diluted suspension of viable, stationary phase cells (viable count 20 cells) was added to 200 μl of LMM supplemented with 0.5 % w/v L-lactate and containing 2 μl of each fraction (from a different experiment to that shown in parts A and B) in 5-10 replicates in the Bioscreen instrument. The apparent lag phase was estimated by extrapolating the exponential growth line to the abscissa.

Figure 4: Effect of purified RP-factor on *M. luteus*.

A. Concentration dependence of RP-factor activity for resuscitation: resuscitation of dormant cells with different concentrations of RP-factor. 10 μl of a diluted suspension of starved cells (CFU 3.10^6 cells.ml^{-1}, total count 5.10^9 cells.ml^{-1}) was added to 200 μl of LMM supplemented with 0.5 % w/v L-lactate, 0.05% yeast extract and RP-factor in concentrations shown in 5-10 replicates in the Bioscreen instrument. For details see Materials and Methods. **B**. Growth of washed cells. Stationary phase cells of *M. luteus* grown in LMM were washed five times by suspension and centrifugation in LMM from which lactate had been omitted. Bacteria were finally suspended in the same medium by repeatedly passing them through a syringe, diluted and inoculated into a 20 ml flask containing LMM or LMM plus 31 pM RP-factor. The initial cell density was 250 viable cells per ml and incubation was at 30°C with intensive shaking. Growth was monitored by plating 0.1ml samples on plates containing broth E solidified with agar.

Figure 5: Detection of RP-factor-like genes in *Micrococcus luteus*, *Mycobacterium smegmatis* and *Streptomyces rimosus*.

	Part A	Part B	Part C
	M. luteus	*M. luteus*	
	Lane 1	λ*Bst*EII	λ*Pst*I	λ*Pst*I
	Lane 2	*Cla*I	*Xho*I	*S. rimosus Xho*I
5	Lane 3	*Sal*I	*Stu*I	*S. rimosus Stu*I
	Lane 4	*Sac*II	*Sma*I	*S. rimosus Sma*I
	Lane 5	*Pst*I	*Pvu*II	*S. rimosus Pvu*II
	Lane 6	*Nco*I	*Pst*I	*S. rimosus Pst*I
	Lane 7	*Nhe*I	*Kpn*I	*S. rimosus Bam*HI
10	Lane 8	*Mlu*I	*Bam*HI	*M. smegmatis Xho*I
	Lane 9	*Aat*II	λ*Pvu*II	*M. smegmatis Stu*I
	Lane 10	λ*Pst*I		*M. smegmatis Sma*I
	Lane 11			*M. smegmatis Pvu*II
	Lane 12			*M. smegmatis Pst*I
15	Lane 13			*M. smegmatis Bam*HI
	Lane 14			λ*Pvu*II

Figure 6 : Effect of *M. luteus* RP-factor on the growth of *Mycobacterium smegmatis* (**A**) and *Mycobacterium bovis* (**B**) in batch culture as observed turbidimetrically. *M.* 20 *smegmatis* was grown in broth E, to which was added RP-factor at 31 pMol/L. Cells were inoculated at a level of *circa* 200 per well, and growth was monitored in the Bioscreen instrument. *M. bovis* was grown in Sauton medium, as described in the Materials and Methods section, to which RP-factor (620 pMol/L) was either added or not. The inoculum was *circa* 1.10^5 cells.ml^{-1}, and the OD shown is the average of 10 25 separate determinations of 10 separate tubes.

Figure 7: A: Purification of His-tagged RP-factor. RP-factor was expressed in *E. coli* HSM174(DE3) and purified as described *infra*. Shown is the SDS-PAGE profile of fractions following Ni^{2+}-chelation chromatography. The molecular weight (kDal) 30 markers (SIGMA) were bovine serum albumin (67), ovalbumin (43), glyceraldehyde 3-phosphate dehydrogenase (36), carbonic anhydrase (30), soya bean trypsin inhibitor

(20.1), and lactalbumin (14.4). Lane: 1, markers; 2, crude extract from *E. coli* containing pET19b vector; 3, crude extract from *E. coli* containing pRPF1; 4, purified recombinant RP-factor.

B: Reduction of the apparent lag phase of viable cells of *M. luteus* by purified recombinant RP-factor. For experimental details see the legend for Figure 3C. A dilution factor of 10^0 corresponds to 33 ìg RP-factor/ml.

C: Stimulation of the growth of washed cells of *M. luteus* by purified recombinant RP-factor. Stationary phase cells of *M. luteus* grown in LMM were washed 5 times by suspension and centrifugation in LMM from which lactate had been omitted. Bacteria were finally suspended in the same medium by repeatedly passing them through a syringe, diluted, and inoculated into a 20 ml flask with LMM or LMM in the presence of RP-factor (230 pMol/L). The initial cell density was ca. 10^2 viable cells per ml and incubation was at 30∘C with intensive shaking. Growth was monitored by plating 0.1 ml samples on plates containing nutrient broth E solidified with agar.

Figure 8: A: Anti-RP-factor serum inhibits the growth of *Micrococcus luteus*. Bacteria were inoculated at an initial density of $5x10^5$ per ml into lactate minimal medium (LMM) and the OD_{600nm} was monitored at intervals. Growth of the cultures was monitored over 140 hours at intervals. The samples labelled LMM + Ab and LMM + control Ab contain equivalent amounts of immune and pre-immune serum, respectively. Immune serum (Ab) and pre-immune serum (control Ab) were employed at a 1:1000 dilution.

B: RP-factor overcomes the inhibitory effect of anti-RP-factor serum on growth of *Micrococcus luteus*. Bacteria were inoculated at an initial density of 10^7 cells per ml and growth was monitored by measuring the OD_{600nm} at intervals. Immune serum (Ab) and pre-immune serum (control Ab) were employed at a 1:1000 dilution and RP-factor was added at a final concentration of 50 ng/ml.

Figure 9: Part A. Blocked alignment of nine RP-factors (as explained *infra*, MtubZ94752 may be a cognate receptor). Areas of sequence identity/similarity are indicated by the shaded areas. The *S. coelicolor* gene product shown is a fragment.

Part B. Schematic showing the domain structure of some gene products in the RP-factor family.

Figure 10: Effect of recombinant RP-factor on growth of *M. tuberculosis* in Sauton medium. Sauton medium containing 0.05% Tween-80 and 100$_{\mu}$Mol/L Na oleate + 10% (v/v) supplement (which contains, per litre, 50g bovine serum albumin, 20g glucose, 8.5g NaCl) was inoculated to an initial cell density of $31x10^3$ cfu/ml (viable count determined by plating on agar-solidified Middlebrook 7H9 medium containing 10% v/v supplement, composition as detailed above) [total count by microscopy = 10^6 cells per ml] with a 2.5 month-old culture of *M. tuberculosis* strain H37Ra grown in the same medium. Growth of tube cultures at 37∘C was measured by determining the OD_{600nm} at intervals for 28 days. The undiluted concentrations of the RP-factors, Rpf (*M.luteus*) and Rpf2 (*M. tuberculosis*), employed for these experiments were ca. 10$_{\mu}$g/ml.

Examples

Material And Methods

Organisms and media.

Micrococcus luteus NCIMB 13267 (previously described as "Fleming strain 2665") was grown aerobically at 30˚C in shake flasks in lactate minimal medium (LMM) containing L-lactate as described previously. When the culture had reached stationary phase agitation was continued at 30˚C for up to 2 months. Cultures were then held aerobically at room temperature without agitation for period for up to a further 2-3 months. The apparent initial viability of these cultures at this point (measured by comparing the plate count with the microscopic count) was less than 10^{-3}.

Mycobacterium smegmatis ("fast" strain, All-Russia State Institute for Contol of Veterinary Preparations, Moscow) was grown in either Sauton medium or nutrient broth E (LabM). Overnight pre-cultures were used to inoculate cultures to an initial density of 10^3 cells/ml. *Mycobacterium bovis* (BCG), *Mycobacterium tuberculosis* H37RV and *Mycobacterium avium* were grown in Sauton medium.

M. luteus *Spent medium preparation.*

Supernatant was obtained after the centrifugation of late logarithmic phase *M. luteus* cultures (200-1000 ml) grown in lactate minimal medium or in the same medium in which lactate was replaced by succinate plus 0.01% yeast extract from which macromolecules had been removed by dialysis. The inoculum consisted of 2% of cells grown in rich medium (Broth E, LabM) and then washed in LMM lacking lactate. The supernatants were passed through a 0.22 μm filter (Whatman) before use.

M. luteus *Cell viability by plating.*

Plates consisting of 1.3% Nutrient Broth E (LabM) or lactate minimal medium were used. Cell dilutions were made in quadruplicate with centrifuged and autoclaved spent medium taken from the starved culture. Plates were incubated at 30°C for 3-5 d.

M. luteus *Cell viability by MPN.*

The MPN assay was performed in a Bioscreen C optical growth analyzer (Labsystems, Finland) using lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract as a resuscitation medium. Dilutions of starved cells were made as described. 10 μl of each dilution (5-10 replicates) were added to a well containing 200 μl of either lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract or the same medium with fraction tested (2-20 μl). Growth (optical density) was monitored using a 600 nm filter. Plates were incubated at 30°C with intensive continuous shaking. The overall measurement period was 120h, each well being measured hourly.

The fractions obtained after chromatography were dialysed against elution buffer 2 (see below), diluted in resuscitation medium in various proportions (1:10, 1:100, 1:500, 1:1000, 1:5,000, 1:10,000) and filtered through 0.22 μm Gelman filters before testing. The calculation of the MPN was based on published Tables.

Total cell counts

Unstained cells were counted with a phase-contrast microscope and an improved Neubauer counting chamber. In long-term experiments with mycobacteria, organisms were stained with Ziehl-Neelsen reagent before counting.

Chromatography

Pre-wetted DEAE cellulose was added to culture supernatant (1:10 v/v) and incubated at 4°C for 1h with slow stirring. The cellulose was loaded into a column, and washed with 5 volumes of buffer 1 consisting of 10mM Tris-Cl, 1mM EDTA, 1mM DTT, 10% (v/v) glycerol, pH 7.4 with 10mM KCl. The column was eluted stepwise with 2-3 bed volumes of 0.3M KCl in buffer 1. The fraction obtained was slowly diluted with buffer 1 on ice to give a final KCl concentration of 0.08M. Forty column volumes of this fraction was then loaded onto a DEAE-sepharose fast flow column (1 part of sepharose pre-equilibrated with buffer 1 containing 0.08M KCl). The column was washed with 5 bed volumes buffer 1 containing 0.08M KCl and eluted stepwise with 3 volumes of 0.25M KCl in buffer 1. The fraction obtained was again slowly diluted with buffer 1 on ice to a final KCl concentration of 0.08M, filtered through a 0.22 μm Gelman filter and loaded onto a Mono Q column (model HR5/5, pre-packed, Pharmacia) equilibrated with buffer 2 consisting of 10mM Tris-Cl, 10% glycerol, pH 7.4 containing 0.08M KCl. The Mono Q column was eluted by a linear gradient from 0.08 M to 0.28 M KCl in buffer 2 (the total volume of the elution was 20 ml). The flow rate and fraction size were 1 ml/min and 1ml/tube respectively. All manipulations except the Mono Q chromatography step were performed at 4°C. The fractions obtained were dialysed against 10 mM Tris-Cl containing 10% glycerol (dialysis is important for the retention of activity) and stored at

4°C for up to 5 days without loss of activity. For prolonged storage in a deep freeze, fractions were dialysed in the same way and glycerol added to a final concentration of 20-30% w/v. The protein content in purified preparations was estimated by tryptophan fluorescence using lysozyme as a standard.

Trypsin treatment:

Trypsin was added to the active, dialysed fraction obtained from the mono Q column and diluted by LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract (1:100) (the final concentration of trypsin was 50 μg/ml). The mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of trypsin inhibitor (100 μg/ml). In control experiments trypsin inhibitor was added to the mixture (100 μg/ml) prior to incubation.

PAGE electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli. Chromatographic fractions were dialysed against 10mM Tris HCl, pH 7.4 for 4-5 h, dried in a speed-vacuum apparatus (1.5h), dissolved in sample buffer (Sigma, S-3401), loaded onto 15% acrylamide gel and run at a constant voltage of 200V . The gel was stained with colloidal Coomassie G (Sigma).

Chemicals.

Nutrient Broth E, yeast extract and agar were obtained from Lab M, whilst L-lactate (Li salt), succinate, trypsin, soybean trypsin inhibitor and DEAE-Sepharose fast flow were obtained from Sigma. DEAE cellulose DE52 was obtained from Whatman, and Mono S and Mono Q from Pharmacia. Other chemicals were of analytical grade and were obtained from Sigma or BDH.

DNA manipulations.

Protein microsequence data from the N-terminus (**ATVDTWDRLAE**exSNGTxD) and an internal peptide (VG**GEGYPHQA**SK) obtained from the purified RP-factor were used to design two oligonucleotides, denoted A1 [GCSACSGTSGACACSTGGGACCGSCTSGCSGAG] and A2 [GCYTGRTGIGGRTAICCYTCICC], respectively. Taq polymerase was employed under standard conditions to amplify a 147 bp PCR product from *M. luteus* DNA with these primers. The PCR product obtained from *M. luteus* DNA with these two primers was labelled with digoxygenin and used as a probe for Southern hybridisation experiments. *Sma*I-digested genomic DNA was size-fractionated by agarose gel electrophoresis and circa 1.4 kbp fragments were cloned in pMTL20 and established in *E. coli* strain DH5á. Two recombinant plasmids carrying the desired insert were detected by hybridisation, confirmed by PCR using oligonulceotides A1 and A2, and one of them was manually sequenced on both strands using the dideoxy chain termination method.

Standard procedures were employed to isolate DNA from *M. luteus* and *M. smegmatis*. *Streptomyces rimosus* DNA was kindly supplied by Dr. D. Hranueli. Southern hybridisations with *M. smegmatis* and *S. rimosus* DNA were initially carried out under non-stringent conditions (0.5 SSC, 37∘C). Stringent conditions (0.1 SSC, 65∘C) were subsequently employed for screening an ordered cosmid library of *Streptomyces coelicolor* A3(2) DNA.

Purification of RP-factor

RP-factor purified from culture supernatants of cells grown in lactate minimal medium, according to the protocol described in Materials and Methods, revealed the presence of a significant amount of polymeric material eluted from all types of columns used, which inhibited both the resuscitation of dormant cells and the growth of viable cells of *M. luteus*. Moreover, elevated concentrations of this material could even cause the lysis of cells (not shown). This inhibitory material appears to be a polymer derived from lactate,

as lactate-containing LMM stored for 10 hours at room temperature without cells and subjected to the same procedure of purification revealed inhibitory properties similar to those of this spent medium. To avoid this problem we replaced lactate in the growth medium with succinate, although for good growth it proved necessary to add a small amount (0.01 % w/v) of yeast extract dialysed to remove macromolecules.

Using succinate-grown cultures, the active fraction was purified by a combination of anion exchange media (see Material and Methods). The final activity was eluted at around 180 mM KCl from a linear KCl gradient (from 0.08 to 0.28M KCl) on a MonoQ column in 3 adjacent fractions (Fig. 3). It is worth mentioning that it proved important to dialyse the fractions before testing their activity because some fractions were inactive before dialysis. Active fractions did not change their resuscitation activity after dilution up to 400 times (v/v).

Interestingly, those fractions which were active in causing resuscitation could also increase the growth rate of viable cells.

The resuscitation-promoting material from the final purification step was checked by SDS-PAGE. The final product (Fig. 3C) proved to consist of a single protein with a molecular weight estimated to be ca 16kD. All active fractions consist of single band with maximum content of protein in fraction N9.

Cloning of the RP-factor gene

Two primers were designed from protein microsequence data obtained for the N-terminus of the purified RP-factor and for an internal peptide. They were used to amplify a 147 bp fragment of *M. luteus* DNA, which was cloned and sequenced. The complete gene was then obtained by a combination of inverse PCR using oligonucleotides G1 and G2 and isolation of a 1.4 kbp *Sma*I genomic restriction fragment. Sequencing revealed that the original PCR product was part of a gene capable of encoding a protein having a signal sequence (Fig. 2A). The predicted size of the secreted form of the gene product is

19,148 Dal, and its predicted N-terminal amino acid sequence agrees with the protein microsequence data, including residues that were not used in primer design (Fig. 2A). The fact that the predicted gene product is larger than the RP-factor purified from culture supernatants suggests that it may, for example, be secreted as a precursor which is converted to its biologically active form upon contact with its cognate receptor/convertase.

Identification of RP-factor homologues

A BLAST search was undertaken using the predicted amino acid sequence of the ORF from *M. luteus* as query. Seven genes with substantial similarity have been sequenced previously. Five are found in *M. tuberculosis* and two in *Mycobacterium leprae* (Fig. 1A). One or more gene products in each organism appear to have a secretory signal sequence (underlined in Fig. 1A). The functions of the predicted products of these mycobacterial genes are unknown; they were found by genome sequencing projects. The BLAST search also revealed similarity between residues 126-220 of the RP-factor and a conserved segment of the (major extracellular) p60 proteins that have been implicated in adherence of *Listeria* spp. to 3T6 mouse fibroblasts suggesting, perhaps, a possible role for the RP-factor or a proteolytic product thereof in adhesion in *M. luteus* (Fig. 1E).

In common with *M tuberculosis* and *M. leprae*, *M. luteus* contains a second gene similar to that encoding the RP-factor. Southern hybridisation experiments, using DNA samples cleaved with a range of different restriction enzymes, and the cloned 147 bp fragment as probe (Figs. 5A & B), reveal two hybridising bands. The stronger hybridisation signal arises from the gene encoding the secreted RP-factor. The other gene may correspond to one of the other mycobacterial genes identified above.

Southern hybridisation experiments, using the 147 bp fragment as probe, as well as PCR experiments, using two oligonucleotides based on highly conserved amino acid motifs as primers, indicate that genes encoding proteins similar to the RP-factor are of

widespread occurrence, at least throughout Gram-positive bacteria whose DNA has a high G+C content. Similar genes are detectable by either or both of these methods in all six *Streptomyces* species we have tested, including *Streptomyces rimosus* (Fig. 5C) as well as in other mycobacteria, including *Mycobacterium smegmatis* (four similar genes - Fig. 5C), *Mycobacterium bovis* (BCG) and *Corynebacterium glutamicum* (2 similar genes).

Domain structure

The sequence information shows that the RP-factor gene and all of its mycobacterial homologues share a secretory signal sequence and a particularly highly conserved, ca. 70-residue segment. One (MTubZ94752) also has a membrane anchoring motif. The conserved 70-residue segment is a candidate for a signalling domain. Most of this segment is weakly hydrophilic (Kyte-Doolittle) and is predicted to form amphipathic á-helical (Garnier-Robson; Chou-Fasman) or β-sheet regions (Eisenberg). Overall, the segment has a low surface probability (Emini). The C-terminal section, by contrast, is much less highly conserved and might be considered a better candidate for determining localization or specificity (i.e. be a cellular compartment-targeting or specificity-determining domain). By analogy with other protein signalling systems (e.g. many pro-hormones in animals, and systemin in plants) it is possible that the proximate signalling molecule is a proteolytically cleaved product.

Two acidic residues, D7 & E13 (numbering according to the *M. luteus* secreted protein), within this segment are absolutely conserved. The KAEQIKRAE segment (residues 51-59) represents an island of particularly high surface probability. These elements may form part of functional domains within the RP-factor protein.

The conserved domain contains four conserved tryptophan residues (one of which is in a region of high surface probability DTWDR - residues 4-8). In the complex between human growth hormone and its first bound receptor, interactions involving two surface-located tryptophan residues in the receptor account for more than 75% of the binding

free energy of the complex (Clackson and Wells, Science 267, 383-386, 1995). The two conserved cysteine residues may form a disulphide bridge.

Alignments showing the domain structures of the various proteins are shown in Figs.9A and 9B.

RP-factor activity

As well as resuscitating dormant cells, the purified RP-factor from *M. luteus* has been tested for growth-stimulatory activity against *M. luteus* and several other organisms. It strongly stimulates the growth *of M. luteus* and *M. smegmatis* and it appears to have weaker activity on *M. tuberculosis*, *M. bovis* (BCG) and *M. avium* (see Fig. 6). In all cases, there is a shortening of the apparent lag phase in batch culture (see Figs. 3D, 4B, 6B and Table 1). The factor is active in poor media and in poor media supplemented with yeast extract and it loses activity after boiling or treatment with trypsin.

When ca. 40 pMol/L RP-factor was added to washed cells of *Mycobacterium smegmatis*, growth occurred after 20-24 hr, whereas the control lacking RP-factor showed no growth after 6 days. Experiments with slowly growing mycobacteria yielded similar results. Growth of *M. bovis* (BCG) was also strongly stimulated by 40 pMol/L RP-factor: growth occurred after 14 days whereas the control lacking RP-factor showed no growth after 90 days. Finally, RP-factor also stimulated the growth of *Mycobacterium tuberculosis*, *Mycobacterium smegmatis, Mycobacterium avium* and *Mycobacterium kansasii* (see Table 1).

Table 1. Purified *M. luteus* RP-factor stimulates growth of mycobacteria

Organism	Bacterial growth[$] RP-factor omitted	RP-factor added
Mycobacterium tuberculosis H37Ra	1.3 ± 1.9 (5)	110 ± 32 (5)
Mycobacterium tuberculosis H37Rv	1.5 ± 2 (4)	45 ± 28 (4)
Mycobacterium avium	0 (3)	>300 (3)
M. bovis (BCG)	0 (5)	54 ± 38 (5)
*M. smegmatis**	0 (8)	225 ± 44 (8)
Mycobacterium kansasii	2.5 ± 2.5 (3)	90 ± 77 (3)

[$]Growth was estimated microscopically (magnification times 600) after 14 days of incubation; ca. 50 µl of each culture was fixed, stained using Ziehl-Neelsen reagent and counted. Values in the body of the Table are average numbers of cells in a microscope field (10-20 fields counted) ± standard deviation with the number of determinations in parentheses. RP-factor (after elution from the Mono Q column and dialysis) was used at a concentration of circa 40 pMol/L; activity was lost after either trypsin treatment, heating (autoclaving) or filtration through a 12 kDal cutoff membrane.

*Washed cells of *M. smegmatis* were used for this experiment.

Isolation and characterisation of the gene encoding the second homologue from *M. luteus*

A combination of inverse PCR using oligos G1 and G2 (see Fig. 2A) as primers, and cloning of suitably sized genomic restriction fragments, can be employed to isolate the gene encoding the second homologue from *M. luteus*. The sequence of the gene can then be determined, taking care to eliminate any possible PCR errors by analysis of genomic clones and direct sequencing of PCR fragments obtained by combining the products of multiple, independent PCR reactions. Comparative sequence analyses of the proteins from *M. luteus*, *M. leprae* and *M. tuberculosis* can then be used to refine predictions

concerning residues, sequence motifs and structural motifs which may be important for biological function.

Over-expression and purification of *M. luteus* and *M. tuberculosis* gene products in *E. coli*

PCR primers can be designed, incorporating suitable restriction sites such that sequences encoding the secreted forms of the *M. luteus* and the *M. tuberculosis* RP-factors can be amplified and inserted, in the correct reading frame, into commercially available plasmids (pET or pCAL vectors). The PCR-amplified fragments can first be cloned in a pBluescript KS II vector (Stratagene) so that their entire sequence can be verified, to eliminate possible PCR errors. (This material can also be employed for site-directed mutagenesis - *vide infra*.) The pET or pCAL constructs can then be employed to obtain controlled expression of large quantities of histidine- or calmodulin binding peptide-tagged proteins that can be purified, essentially to homogeneity, in a single step. Finally, the tags used in protein purification can be removed (using enterokinase or thrombin, as appropriate).

Expression of RP-factor from *Micrococcus luteus* in *E. coli*

Two primers [5'-GTCAGAATTCATATGGCCACCGTGGACACCTGGG-3'] and [5'-TGACGGATCCTATTAGGCCTGCGGCAGGACGAG-3'] were employed to amplify (5 cycles of 30s at 94∘C, 30s at 60∘C, 30s at 72∘C, followed by 15 cycles of 30s at 94∘C, 60s at 72∘C) the RP-factor coding sequence (i.e. lacking the signal sequence) from the cloned 1.4 kbp *Sma*I fragment of genomic DNA. It was first established in *E. coli* DH5á as a 567 bp *Eco*RI-*Bam*HI fragment in pMTL20 and then excised as a 562 bp *Nde*I - *Bam*HI fragment, inserted into pET19b (Novagen) and re-established in *E. coli* DH5á. The sequence of the PCR product and vector-insert junction in this plasmid, denoted pRPF1, was verified. RP-factor was expressed from RPF1 after transforming it into *E. coli* HSM174(DE3). The protein, containing a His_{10}-tag at the N-terminus, was isolated by sonicating bacteria, previously grown to an OD_{600nm}=0.6 and induced with

0.4 mM IPTG for 4 h, in a modified binding buffer (MBB - 5mM imidazole pH7.9/0.5M NaCl/20mM Tris-HCl/8M urea) containing 5 mM DTT and 2 mM EDTA. After low speed centrifugation, low MW compounds, including EDTA and DTT, were removed by elution through a Sephadex G10 column pre-equilibrated with MBB. A Ni^{2+}-chelation column (Ni^{2+}-coordinated iminodiacetic acid immobilized on Sepharose 6B), was loaded with the G10 eluate, washed with 20 vol MBB and then successively eluted with four 10 vol aliquots of MBB containing 0.01 M, 0.05 M, 0.2 M and 1 M imidazole, respectively. The column was finally eluted with strip buffer (20 mM Tris-HCl, pH 7.9/100 mM EDTA/0.5 M NaCl). Monoclonal anti-(polyHis) antibodies (Sigma, clone His-1) were employed for immunoblot analysis of fractions subjected to SDS PAGE electrophoresis and electroblotted using standard methods. Fractions were dialysed against buffer 2 and assayed for biological activity as indicated above.

Analysis of recombinant RP-factor

The coding sequence corresponding to the secreted form of RP-factor, starting at residue A_{39}, was inserted into pET19b to generate plasmid pRPF1 (*vide infra*). Extracts of IPTG-induced *E. coli* strain HSM174(DE3) containing pRPF1 were challenged with a poly-His antibody. A strong signal was associated with a protein (apparent size 29 kDal, predicted size 22 kDal) which was eluted from the affinity column by 1M imidazole (Fig. 7A). The His-tagged protein from HSM174(DE3) reduced the apparent lag phase of viable cells of *M. luteus*, whereas the control (material eluted from the same column under the same conditions when an extract from cells containing plasmid vector only was applied) showed no activity (Fig. 7B). The association of biological activity with the recombinant protein, produced in *E. coli* containing pRPF, and the absence of biological activity in the isogenic control containing pET19b, demonstrates unequivocally that the active molecule is indeed a product of the *rpf* gene.

Antibody preparation

A rabbit was immunized three times at one week intervals using recombinant RP-factor (the recombinant protein prepared as described above). The protein was administered at 300 μg of protein per injection in incomplete Freud's adjuvant (0.5 ml protein and 0.5 ml adjuvant) Blood was collected before administration was started and on the 11th day after the last injection. The immunoglobulin fraction was obtained by standard procedures using PEG. Antibodies were additionally purified on a protein G-superose column according to the standard (Pharmacia) protocol. The final protein concentration was adjusted spectrophotometrically to 1 mg/ml.

Alternatively, monoclonal antibodies can be produced using established techniques.

Use of anti-RP-factor antibody to inhibit bacterial growth

Micrococcus luteus was inoculated at an initial density of $5x10^5$ per ml into lactate minimal medium (LMM) and the OD_{600nm} was monitored at intervals. Growth of the cultures was monitored over 140 hours, and the presence of the anti-RP-factor serum (prepared as described above under "Antibody preparation") completely inhibited bacterial growth (see Figure 8).

Expression of a *M. tuberculosis* RP-factor in *E. coli*

Two primers [5'-ATCAGAATTCATATGGACGACATCGATTGGGACGC-3'] and [5'-CGCAGGATCCCCTCAATCGTCCCTGCTCC-3'] were employed to amplify (5 cycles of 30s at 94∘C, 30s at 58∘C, 30s at 72∘C, followed by 25 cycles of 30s at 94∘C, 60s at 72∘C) the RP-factor coding sequence (i.e. lacking the signal sequence) from *M. tuberculosis* H37Rv genomic DNA. The PCR product was first established in *E. coli* DH5a as a 336 bp *Eco*RI-*Bam*HI fragment in pMTL20 and then excised as a 331 bp *Nde*I - *Bam*HI fragment, inserted into pET19b (Novagen) and re-established in *E. coli* DH5a. The sequence of the PCR product and vector-insert junction in this plasmid,

denoted pRPF2, was verified. The *M. tuberculosis* RP-factor was expressed from pRPF2 after transforming it into *E. coli* HSM174(DE3). The protein, containing a His_{10}-tag at the N-terminus, was isolated by sonicating bacteria, previously grown to an OD_{600nm} = 0.9 and induced with 0.4 mM IPTG for 4 h, in binding buffer (BB - 5mM imidazole pH7.9 / 0.5M NaCl / 20 mM Tris-HCl / 8M urea). After low speed centrifugation, a Ni^{2+}-chelation column (Ni^{2+}-coordinated iminodiacetic acid immobilised on Sepharose 6B), was loaded with the supernatant, washed with 20 vol BB, 20 vol BB containing 100 mM imidazole, and then eluted with 10 vol BB containing 0.5 M imidazole. Additional purification was achieved by MonoQ column chromatography (*vide infra*, save that the salt gradient was from 0.1 M to 1M NaCl). Monoclonal anti-(polyHis) antibodies (Sigma, clone His-1) were employed for immunoblot analysis of fractions subjected to SDS PAGE electrophoresis and electroblotted using standard methods. Fractions were dialysed against buffer 2 and assayed for biological activity as indicated above.

Analysis of a recombinant *M. tuberculosis* RP-factor

The coding sequence corresponding to the secreted form of the *M. tuberculosis* RP-factor (g1655671; acc. no. Z81368), starting at residue D_{50}, was inserted into pET19b to generate plasmid pRPF2 (*vide infra*). Extracts of IPTG-induced *E. coli* strain HSM174(DE3) containing pRPF2 were challenged with a poly-His antibody. A strong signal was associated with a protein which was eluted from the affinity column by 0.5M imidazole. The histidine-tagged protein from HSM174(DE3) caused a sl;ight but significant enhancement of the growth of *M.tuberculosis* H37Rv, as shown in Fig. 10. It also stimulated the growth of *M. luteus* in LMM. The control culture attained a final OD_{600nm} of 1.0, whereas cultures containing the RP-factor (1:100,000 dilution) attained a final OD_{600nm} of between 2.0 and 6.0.

Effect of *M. luteus* RP-factor on growth of *Mycobacterium tuberculosis* cells isolated from macrophages

In three independent experiments, dormant/latent *M. tuberculosis* cells isolated from cultured murine peritonial macrophages were resuscitated by the *M. luteus* RP-factor. The total number of *M. tuberculosis* cells in the heterogeneous suspension obtained from murine macrophages was determined microscopically. The viable cell count was determined by plating on agar-solidified Sauton medium containing 10% (v/v) supplement (which contains, per litre, 50 g bovine serum albumin, 20g glucose, 8.5g NaCl) or by the MPN method, using liquid Sauton medium containing 10% (v/v) supplement (see above).

The viable count (MPN) of these cell suspensions was enhanced between 25 and 2,500 times by the presence of the *M. luteus* RP-factor (added at a final concentration of 10 ng/ml) (see Table 2). All values in the body of the table are numbers of bacteria per ml suspension

Peritoneal macrophages were obtained from white mice (wild type) by a standard protocol. Infection of macrophages by *M. tuberculosis* "Academiya" (laboratory strain) was performed *in vivo* by intraperitonial injection of 10^6 cells (total count) per mouse followed by incubation for 6 days (1st passage). For the second and third passages macrophage cells in monolayers were infected using *M. tuberculosis* cells isolated from macrophages from the previous passage.

TABLE 2: Effect of *M. luteus* RP-factor on growth of *Mycobacterium tuberculosis* cells isolated from macrophages

Experiment	Total count [x] (determined microscopically)	Viable count (determined by plating)	Viable count (MPN)	MPN in presence of RP-factor
I	$10^6 > x > 10^5$	90	70	4.10^3
II	$10^6 > x > 10^5$	9	40	1.10^5
III	2.10^6	<1	<1	24.10^3

Macrophages were grown as a monolayer on plastic petri dishes (10^6 cells/5 cm^2) in standard RPMI medium containing gentamicin and penicillin (10ig/ml, each) under standard conditions (CO_2/O_2 mixture in a 37°C incubator). *M. tuberculosis* cells were recovered from macrophages by passing them repeatedly through a thin syringe needle. Macrophage cell debris was removed by low speed centifugation and *M. tuberculosis* cells were then collected by centrifugation at higher speed.

Effect of *yabE* and *yocH* knockout mutations on growth of *Bacillus subtilis*

The entire *yabE* coding region together with flanking sequences was amplified from *B. subtilis* genomic DNA using primers D11 [5'-GAAGAGAATTCCTTCCATCACGA-3'] and D12 [5'-CCAAACGAATTCGGTCAATCAC-3'] as a 1803 bp product. A 1186 bp *Hin*dIII-*Bcl*I fragment encompassing the 3' end of the coding sequence was excised from the PCR product, ligated with *Hin*dIII + *Bam*HI-digested pMTL20, and used to transform *E. coli* strain DH5á with selection for ampicillin-resistance. Plasmid pYABE was isolated from one of the transformants. A 763 bp *Hin*dIII-*Bam*HI fragment from entirely within the *yabE* coding sequence was excised from the pYABE, ligated with *Hin*dIII + *Bam*HI-digested pMUTIN4, an integrating plasmid that may be employed for generating knockout mutations in *B. subtilis* (Edwards & Errington, 1997, Molecular Microbiology, 24, 905-915) and used to transform *E. coli* strain XL1-Blue with

selection for ampicillin-resistance. Plasmid pYAB2, containing an internal segment of the *yabE* coding sequence, was isolated from one of the transformants. A 1207 bp *Hin*dIII-*Eco*RI fragment encompassing the 3' end of the *yabE* coding sequence was excised from pYABE, ligated with *Hin*dIII + *Eco*RI digested pMUTIN4 and used to transform *E. coli* strain XL1-Blue with selection for ampicillin-resistance. Plasmid pYAB3, containing the 3' end of the *yabE* coding sequence, was isolated from one of the transformants.

The entire *yocH* coding region together with flanking sequences was amplified from *B. subtilis* genomic DNA using primers D10 [5'-GCAAGGATCCCAGACTAAAAAAACAG-3'] and D9 [5'-ATCAGGATCCATATTATTAGTTTAAGA-3'] as a 1145 bp product. A 358 bp *Hpa*I fragment from entirely within the *yocH* coding sequence was excised from the PCR product, ligated with *Sma*I-digested pMTL20, and used to transform *E. coli* strain XL1-Blue with selection for ampicillin-resistance. Plasmid pYOC2a, containing an internal segment of the *yocH* coding sequence, was isolated from one of the transformants. The insert in this plasmid was then excised from pYOC2a as a 385 bp *Eco*RI-*Hin*dIII fragment and inserted into pMUTIN4, to yield pYOC2. A 307 bp *Hin*dIII-*Bam*HI fragment encompassing the 3' end of the *yocH* coding sequence was excised from the 1145 bp PCR product, ligated with *Hin*dIII + *Bam*HI digested pMUTIN4, and used to transform *E. coli* strain DH5á with selection for ampicillin-resistance. Plasmid pYOC3, containing a DNA segment encompassing the 3' end of the *yocH* coding sequence, was isolated from one of the transformants.

Plasmids pYAB2, pYAB3, pYOC2 and pYOC3 were linearised with *Apa*I, which cleaves once in the pMUTIN4 vector sequences, ligated with T4 DNA ligase and employed to transform *Bacillus subtilis* strain SA253 *nonA nonB leuA8 arg-15* with selection for resistance to erythromycin on a rich nutrient medium (LB + 1 ig Em/ml). Em^R transformants were then picked and verified by Southern hybridization. Using the integrating plasmid as probe, and digesting the chromosomal DNA with *Apa*I, strains harbouring a single copy of the integrated plasmid gave two hybridising bands whereas

the wild type (and any spontaneous Em^R mutants that were present) gave a single hybridising band.

Analysis of the products of transformation with each of the four plasmids indicates that *yabE* and *yocH* gene products are required for growth (at least under certain conditions) in *B. subtilis*.

BACTERIAL PHEROMONES AND USES THEREFOR

Abstract

RP-factors, their cognate receptors, convertases, respective genes and inhibitors or mimetics thereof are described. In particular, antibodies, pharmaceutical compositions and (therapeutic, diagnostic) methods based on the RP-factors and their receptors/convertases are described

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Bacterial Pheromones and Uses Therefor

(iii) NUMBER OF SEQUENCES: 59

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: Not Assigned

(vi) PRIOR APPLIATION DATA:
(A) APPLICATION NUMBER: PCT/GB98/01619
(b) FILING DATE: 03-MAY-1998

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: GB 9711389.8
(B) FILING DATE: 04-JUN-1997

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9811221.2
(B) FILING DATE: 27-MAY-1998

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 362 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Leu Arg Leu Val Val Gly Ala Leu Leu Leu Val Leu Ala Phe Ala
1               5                  10                  15

Gly Gly Tyr Ala Val Ala Ala Cys Lys Thr Val Thr Leu Thr Val Asp
            20                  25                  30

Gly Thr Ala Met Arg Val Thr Thr Met Lys Ser Arg Val Ile Asp Ile
        35                  40                  45

Val Glu Glu Asn Gly Phe Ser Val Asp Asp Arg Asp Asp Leu Tyr Pro
    50                  55                  60

Ala Ala Gly Val Gln Val His Asp Ala Asp Thr Ile Val Leu Arg Arg
65                  70                  75                  80

Ser Arg Pro Leu Gln Ile Ser Leu Asp Gly His Asp Ala Lys Gln Val
                85                  90                  95

Trp Thr Thr Ala Ser Thr Val Asp Glu Ala Leu Ala Gln Leu Ala Met
            100                 105                 110

Thr Asp Thr Ala Pro Ala Ala Ala Ser Arg Ala Ser Arg Val Pro Leu
        115                 120                 125

Ser Gly Met Ala Leu Pro Val Val Ser Ala Lys Thr Val Gln Leu Asn
    130                 135                 140

Asp Gly Gly Leu Val Arg Thr Val His Leu Pro Ala Pro Asn Val Ala
145                 150                 155                 160

Gly Leu Leu Ser Ala Ala Gly Val Pro Leu Leu Gln Ser Asp His Val
                165                 170                 175

Val Pro Ala Ala Thr Ala Pro Ile Val Glu Gly Met Gln Ile Gln Val
            180                 185                 190

Thr Arg Asn Arg Ile Lys Lys Val Thr Glu Arg Leu Pro Leu Pro Pro
        195                 200                 205

Asn Ala Arg Arg Val Glu Asp Pro Glu Met Asn Met Ser Arg Glu Val
    210                 215                 220
```

```
Val Glu Asp Pro Gly Val Pro Gly Thr Gln Asp Val Thr Phe Ala Val
225                 230                 235                 240

Ala Glu Val Asn Gly Val Glu Thr Gly Arg Leu Pro Val Ala Asn Val
                245                 250                 255

Val Val Thr Pro Ala His Glu Ala Val Val Arg Val Gly Thr Lys Pro
            260                 265                 270

Gly Thr Glu Val Pro Pro Val Ile Asp Gly Ser Ile Trp Asp Ala Ile
        275                 280                 285

Ala Gly Cys Glu Ala Gly Gly Asn Trp Ala Ile Asn Thr Gly Asn Gly
    290                 295                 300

Tyr Tyr Gly Gly Val Gln Phe Asp Gln Gly Thr Trp Glu Ala Asn Gly
305                 310                 315                 320

Gly Leu Arg Tyr Ala Pro Arg Ala Asp Leu Ala Thr Arg Glu Glu Gln
                325                 330                 335

Ile Ala Val Ala Glu Val Thr Arg Leu Arg Gln Gly Trp Gly Ala Trp
            340                 345                 350

Pro Val Cys Ala Ala Arg Ala Gly Ala Arg
        355                 360
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 188 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Pro Val Gly Trp Leu Trp Arg Ala Arg Thr Ala Lys Gly Thr Thr
1               5                   10                  15

Leu Lys Asn Ala Arg Thr Thr Leu Ile Ala Ala Ala Ile Ala Gly Thr
            20                  25                  30

Leu Val Thr Thr Ser Pro Ala Gly Ile Ala Asn Ala Asp Asp Ala Gly
        35                  40                  45

Leu Asp Pro Asn Ala Ala Ala Gly Pro Asp Ala Val Gly Phe Asp Pro
    50                  55                  60

Asn Leu Pro Pro Ala Pro Asp Ala Ala Pro Val Asp Thr Pro Pro Ala
65                  70                  75                  80

Pro Glu Asp Ala Gly Phe Asp Pro Asn Leu Pro Pro Pro Leu Ala Pro
                85                  90                  95

Asp Phe Leu Ser Pro Pro Ala Glu Glu Ala Pro Pro Val Pro Val Ala
            100                 105                 110

Tyr Ser Val Asn Trp Asp Ala Ile Ala Gln Cys Glu Ser Gly Gly Asn
        115                 120                 125
```

```
Trp Ser Ile Asn Thr Gly Asn Gly Tyr Tyr Gly Gly Leu Arg Phe Thr
    130                 135                 140

Ala Gly Thr Trp Arg Ala Asn Gly Gly Ser Gly Ser Ala Ala Asn Ala
145                 150                 155                 160

Ser Arg Glu Glu Gln Ile Arg Val Ala Glu Asn Val Leu Arg Ser Gln
                165                 170                 175

Gly Ile Arg Ala Trp Pro Val Cys Gly Arg Arg Gly
            180                 185
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 174 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Ser Glu Ser Tyr Arg Lys Leu Thr Thr Ser Ser Ile Ile Val Ala
1               5                   10                  15

Lys Ile Thr Phe Thr Gly Ala Met Leu Asp Gly Ser Ile Ala Leu Ala
            20                  25                  30

Gly Gln Ala Ser Pro Ala Thr Asp Ser Glu Trp Asp Gln Val Ala Arg
        35                  40                  45

Cys Glu Ser Gly Gly Asn Trp Ser Ile Asn Thr Gly Asn Gly Tyr Leu
    50                  55                  60

Gly Gly Leu Gln Phe Ser Gln Gly Thr Trp Ala Ser His Gly Gly Gly
65                  70                  75                  80

Glu Tyr Ala Pro Ser Ala Gln Leu Ala Thr Arg Glu Gln Gln Ile Ala
                85                  90                  95

Val Ala Glu Arg Val Leu Ala Thr Gln Gly Ser Gly Ala Trp Pro Ala
            100                 105                 110

Cys Gly His Gly Leu Ser Gly Pro Ser Leu Gln Glu Val Leu Pro Ala
        115                 120                 125

Gly Met Gly Ala Pro Trp Ile Asn Gly Ala Pro Ala Pro Leu Ala Pro
    130                 135                 140

Pro Pro Pro Ala Glu Pro Ala Pro Pro Gln Pro Pro Ala Asp Asn Phe
145                 150                 155                 160

Pro Pro Thr Pro Gly Asp Val Pro Ser Pro Leu Ala Arg Pro
                165                 170
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 407 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Ser Gly Arg His Arg Lys Pro Thr Thr Ser Asn Val Ser Val Ala
1               5                  10                  15

Lys Ile Ala Phe Thr Gly Ala Val Leu Gly Gly Gly Gly Ile Ala Met
            20                  25                  30

Ala Ala Gln Ala Thr Ala Ala Thr Asp Gly Glu Trp Asp Gln Val Ala
        35                  40                  45

Arg Cys Glu Ser Gly Gly Asn Trp Ser Ile Asn Thr Gly Asn Gly Tyr
    50                  55                  60

Leu Gly Gly Leu Gln Phe Thr Gln Ser Thr Trp Ala Ala His Gly Gly
65                  70                  75                  80

Gly Glu Phe Ala Pro Ser Ala Gln Leu Ala Ser Arg Glu Gln Gln Ile
                85                  90                  95

Ala Val Gly Glu Arg Val Leu Ala Thr Gln Gly Arg Gly Ala Trp Pro
            100                 105                 110

Val Cys Gly Arg Gly Leu Ser Asn Ala Thr Pro Arg Glu Val Leu Pro
        115                 120                 125

Ala Ser Ala Ala Met Asp Ala Pro Leu Asp Ala Ala Ala Val Asn Gly
    130                 135                 140

Glu Pro Ala Pro Leu Ala Pro Pro Pro Ala Asp Pro Ala Pro Pro Val
145                 150                 155                 160

Glu Leu Ala Ala Asn Asp Leu Pro Ala Pro Leu Gly Glu Pro Leu Pro
                165                 170                 175

Ala Ala Pro Ala Asp Pro Ala Pro Pro Ala Asp Leu Ala Pro Pro Ala
            180                 185                 190

Pro Ala Asp Val Ala Pro Pro Val Glu Leu Ala Val Asn Asp Leu Pro
        195                 200                 205

Ala Pro Leu Gly Glu Pro Leu Pro Ala Ala Pro Ala Asp Pro Ala Pro
    210                 215                 220

Pro Ala Asp Leu Ala Pro Pro Ala Pro Ala Asp Leu Ala Pro Pro Ala
225                 230                 235                 240

Pro Ala Asp Leu Ala Pro Pro Ala Pro Ala Asp Leu Ala Pro Pro Val
                245                 250                 255

Glu Leu Ala Val Asn Asp Leu Pro Ala Pro Leu Gly Glu Pro Leu Pro
            260                 265                 270

Ala Ala Pro Ala Glu Leu Ala Pro Pro Ala Asp Leu Ala Pro Ala Ser
        275                 280                 285
```

```
Ala Asp Leu Ala Pro Pro Ala Pro Ala Asp Leu Ala Pro Pro Ala Pro
    290                 295                 300

Ala Glu Leu Ala Pro Pro Ala Pro Ala Asp Leu Ala Pro Pro Ala Ala
305                 310                 315                 320

Val Asn Glu Gln Thr Ala Pro Gly Asp Gln Pro Ala Thr Ala Pro Gly
                325                 330                 335

Gly Pro Val Gly Leu Ala Thr Asp Leu Glu Leu Pro Glu Pro Asp Pro
            340                 345                 350

Gln Pro Ala Asp Ala Pro Pro Pro Gly Asp Val Thr Glu Ala Pro Ala
        355                 360                 365

Glu Thr Pro Gln Val Ser Asn Ile Ala Tyr Thr Lys Lys Leu Trp Gln
    370                 375                 380

Ala Ile Arg Ala Gln Asp Val Cys Gly Asn Asp Ala Leu Asp Ser Leu
385                 390                 395                 400

Ala Gln Pro Tyr Val Ile Gly
                405
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 155 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Pro Gly Glu Met Leu Asp Val Arg Lys Leu Cys Lys Leu Phe Val
1               5                   10                  15

Lys Ser Ala Val Val Ser Gly Ile Val Thr Ala Ser Met Ala Leu Ser
            20                  25                  30

Thr Ser Thr Gly Met Ala Asn Ala Val Pro Arg Glu Pro Asn Trp Asp
        35                  40                  45

Ala Val Ala Gln Cys Glu Ser Gly Arg Asn Trp Arg Ala Asn Thr Gly
    50                  55                  60

Asn Gly Phe Tyr Gly Gly Leu Gln Phe Lys Pro Thr Ile Trp Ala Arg
65                  70                  75                  80

Tyr Gly Gly Val Gly Asn Pro Ala Gly Ala Ser Arg Glu Gln Gln Ile
                85                  90                  95

Thr Val Ala Asn Arg Val Leu Ala Asp Gln Gly Leu Asp Ala Trp Pro
            100                 105                 110

Lys Cys Gly Ala Ala Ser Asp Leu Pro Ile Thr Leu Trp Ser His Pro
        115                 120                 125

Ala Gln Gly Val Lys Gln Ile Ile Asn Asp Ile Ile Gln Met Gly Asp
    130                 135                 140
```

Thr Thr Leu Ala Ala Ile Ala Leu Asn Gly Leu
145 150 155

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 176 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met His Pro Leu Pro Ala Asp His Gly Arg Ser Arg Cys Asn Arg His
1 5 10 15

Pro Ile Ser Pro Leu Ser Leu Ile Gly Asn Ile Ser Ala Thr Ser Gly
20 25 30

Asp Met Ser Ser Met Thr Arg Ile Ala Lys Pro Leu Ile Lys Ser Ala
35 40 45

Met Ala Ala Gly Leu Val Thr Ala Ser Met Ser Leu Ser Thr Ala Val
50 55 60

Ala His Ala Gly Pro Ser Pro Asn Trp Asp Ala Val Ala Gln Cys Glu
65 70 75 80

Ser Gly Gly Asn Trp Ala Ala Asn Thr Gly Asn Gly Lys Tyr Gly Gly
85 90 95

Leu Gln Phe Lys Pro Ala Thr Trp Ala Ala Phe Gly Gly Val Gly Asn
100 105 110

Pro Ala Ala Ala Ser Arg Glu Gln Gln Ile Ala Val Ala Asn Arg Val
115 120 125

Leu Ala Glu Gln Gly Leu Asp Ala Trp Pro Thr Cys Gly Ala Ala Ser
130 135 140

Gly Leu Pro Ile Ala Leu Trp Ser Lys Pro Ala Gln Gly Ile Lys Gln
145 150 155 160

Ile Ile Asn Glu Ile Ile Trp Ala Gly Ile Gln Ala Ser Ile Pro Arg
165 170 175

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 154 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Thr Pro Gly Leu Leu Thr Thr Ala Gly Ala Gly Arg Pro Arg Asp

```
1               5                   10                  15

Arg Cys Ala Arg Ile Val Cys Thr Val Phe Ile Glu Thr Ala Val Val
            20                  25                  30

Ala Thr Met Phe Val Ala Leu Leu Gly Leu Ser Thr Ile Ser Ser Lys
        35                  40                  45

Ala Asp Asp Ile Asp Trp Asp Ala Ile Ala Gln Cys Glu Ser Gly Gly
    50                  55                  60

Asn Trp Ala Ala Asn Thr Gly Asn Gly Leu Tyr Gly Gly Leu Gln Ile
65                  70                  75                  80

Ser Gln Ala Thr Trp Asp Ser Asn Gly Gly Val Gly Ser Pro Ala Ala
                85                  90                  95

Ala Ser Pro Gln Gln Gln Ile Glu Val Ala Asp Asn Ile Met Lys Thr
            100                 105                 110

Gln Gly Pro Gly Ala Trp Pro Lys Cys Ser Ser Cys Ser Gln Gly Asp
        115                 120                 125

Ala Pro Leu Gly Ser Leu Thr His Ile Leu Thr Phe Leu Ala Ala Glu
    130                 135                 140

Thr Gly Gly Cys Ser Gly Ser Arg Asp Asp
145                 150
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Ile Arg Thr Ala Ala Val Thr Leu Val Ala Ala Thr Ala Leu Gly Ala
1               5                   10                  15

Thr Gly Glu Ala Val Ala Ala Pro Ser Ala Pro Leu Arg Thr Asp Trp
            20                  25                  30

Asp Ala Ile Ala Ala Cys Glu Ser Ser Gly Asn Trp Gln Ala Asn Thr
        35                  40                  45

Gly Asn Gly Tyr Tyr Gly Gly Leu Gln Phe Ala Arg Ser Ser Trp Ile
    50                  55                  60

Ala Ala Gly Gly Leu Lys Tyr Ala Pro Arg Ala Asp Leu Ala Thr Arg
65                  70                  75                  80

Gly Glu Gln Ile Ala Val Ala Glu Arg Leu Ala Arg Leu Gln Gly Met
                85                  90                  95

Ser Ala Trp
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 438 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Met Gly Glu Arg Glu Gly Arg Val Asp Ser Leu Leu Asp Thr Leu Tyr
1               5                  10                  15

Asn Leu Ser Glu Glu Lys Glu Ala Phe Phe Ile Thr Gln Lys Met Lys
            20                  25                  30

Lys Leu Phe Ser Val Lys Leu Ser Lys Ser Lys Val Ile Leu Val Ala
        35                  40                  45

Ala Cys Leu Leu Leu Ala Gly Ser Gly Thr Ala Tyr Ala Ala His Glu
    50                  55                  60

Leu Thr Lys Gln Ser Val Ser Val Ser Ile Asn Gly Lys Lys Lys His
65                  70                  75                  80

Ile Arg Thr His Ala Asn Thr Val Gly Asp Leu Leu Glu Thr Leu Asp
                85                  90                  95

Ile Lys Thr Arg Asp Glu Asp Lys Ile Thr Pro Ala Lys Gln Thr Lys
            100                 105                 110

Ile Thr Ala Asp Met Asp Val Val Tyr Glu Ala Ala Lys Pro Val Lys
        115                 120                 125

Leu Thr Ile Asn Gly Glu Glu Lys Thr Leu Trp Ser Thr Ala Lys Thr
    130                 135                 140

Val Gly Ala Leu Leu Asp Glu Gln Asp Val Asp Val Lys Glu Gln Asp
145                 150                 155                 160

Gln Ile Asp Pro Ala Ile Asp Thr Asp Ile Ser Lys Asp Met Lys Ile
                165                 170                 175

Asn Ile Glu Pro Ala Phe Gln Val Thr Val Asn Asp Ala Gly Lys Gln
            180                 185                 190

Lys Lys Ile Trp Thr Thr Ser Thr Thr Val Ala Asp Phe Leu Lys Gln
        195                 200                 205

Gln Lys Met Asn Ile Lys Asp Glu Asp Lys Ile Lys Pro Ala Leu Asp
    210                 215                 220

Ala Lys Leu Thr Lys Gly Lys Ala Asp Ile Thr Ile Thr Arg Ile Glu
225                 230                 235                 240

Lys Val Thr Asp Val Val Glu Glu Lys Ile Ala Phe Asp Val Lys Lys
                245                 250                 255

Gln Glu Asp Ala Ser Leu Glu Lys Gly Lys Glu Lys Val Val Gln Lys
            260                 265                 270
```

Gly Lys Glu Gly Lys Leu Lys Lys His Phe Glu Val Val Lys Glu Asn
275 280 285

Gly Lys Glu Val Ser Arg Glu Leu Val Lys Glu Glu Thr Ala Glu Gln
290 295 300

Ser Lys Asp Lys Val Ile Ala Val Gly Thr Lys Gln Ser Ser Pro Lys
305 310 315 320

Phe Glu Thr Val Ser Ala Ser Gly Asp Ser Lys Thr Val Val Ser Arg
325 330 335

Ser Asn Glu Ser Thr Gly Lys Val Met Thr Val Ser Ser Thr Ala Tyr
340 345 350

Thr Ala Ser Cys Ser Gly Cys Ser Gly His Thr Ala Thr Gly Val Asn
355 360 365

Leu Lys Asn Asn Pro Asn Ala Lys Val Ile Ala Val Asp Pro Asn Val
370 375 380

Ile Pro Leu Gly Ser Lys Val His Val Glu Gly Tyr Gly Tyr Ala Ile
385 390 395 400

Ile Ala Ala Asp Thr Gly Ser Ala Ile Lys Gly Asn Lys Ile Asp Val
405 410 415

Phe Phe Pro Ser Lys Ser Asp Ala Ser Asn Trp Gly Val Lys Thr Val
420 425 430

Ser Val Lys Val Leu Asn
435

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 288 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Lys Lys Thr Ile Met Ser Phe Val Ala Val Ala Ala Leu Ser Thr
1 5 10 15

Thr Ala Phe Gly Ala His Ala Ser Ala Lys Glu Ile Thr Val Gln Lys
20 25 30

Gly Asp Thr Leu Trp Gly Ile Ser Gln Lys Asn Gly Val Asn Leu Lys
35 40 45

Asp Leu Lys Glu Trp Asn Lys Leu Thr Ser Asp Lys Ile Ile Ala Gly
50 55 60

Glu Lys Leu Thr Ile Ser Ser Glu Glu Thr Thr Thr Thr Gly Gln Tyr
65 70 75 80

Thr Ile Lys Ala Gly Asp Thr Leu Ser Lys Ile Ala Gln Lys Phe Gly

```
                    85                  90                  95

Thr Thr Val Asn Asn Leu Lys Val Trp Asn Asn Leu Ser Ser Asp Met
                100                 105                 110

Ile Tyr Ala Gly Ser Thr Leu Ser Val Lys Gly Gln Ala Thr Ala Ala
        115                 120                 125

Asn Thr Ala Thr Glu Asn Ala Gln Thr Asn Ala Pro Gln Ala Ala Pro
    130                 135                 140

Lys Gln Glu Ala Val Gln Lys Glu Gln Pro Lys Gln Glu Ala Val Gln
145                 150                 155                 160

Gln Gln Pro Lys Gln Glu Thr Lys Ala Glu Ala Glu Thr Ser Val Asn
                165                 170                 175

Thr Glu Glu Lys Ala Val Gln Ser Asn Thr Asn Asn Gln Glu Ala Ser
            180                 185                 190

Lys Glu Leu Thr Val Thr Ala Thr Ala Tyr Thr Ala Asn Asp Gly Gly
        195                 200                 205

Ile Ser Gly Val Thr Ala Thr Gly Ile Asp Leu Asn Lys Asn Pro Asn
    210                 215                 220

Ala Lys Val Ile Ala Val Asp Pro Asn Val Ile Pro Leu Gly Ser Lys
225                 230                 235                 240

Val Tyr Val Glu Gly Tyr Gly Glu Ala Thr Thr Ala Ala Asp Thr Gly
                245                 250                 255

Gly Ala Ile Lys Gly Asn Lys Ile Asp Val Phe Val Pro Glu Lys Ser
            260                 265                 270

Ser Ala Tyr Arg Trp Gly Asn Lys Thr Val Lys Ile Lys Ile Leu Asn
        275                 280                 285
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 320 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Lys Arg Xaa Xaa Ala Val Ile Leu Met Val Ala Val Ile Phe Thr Ile
1                   5                   10                  15

Ile Ser Ser Met Lys Lys Asn Ile Thr Val Asn Ile Asp Gly Lys Thr
            20                  25                  30

Ser Lys Ile Ile Thr Tyr Lys Ser Asn Glu Gly Ser Ile Leu Ser Lys
        35                  40                  45

Asn Asn Ile Leu Val Gly Pro Lys Asp Lys Ile Gln Pro Ala Leu Asp
    50                  55                  60
```

```
Thr Asn Leu Lys Asn Gly Asp Lys Ile Tyr Ile Lys Lys Ala Ile Ser
65                  70                  75                  80

Val Glu Val Ala Val Asp Gly Lys Val Arg Arg Val Lys Ser Ser Glu
                85                  90                  95

Glu Thr Val Ser Lys Met Leu Lys Ala Glu Lys Ile Pro Leu Ser Lys
            100                 105                 110

Val Asp Lys Val Asn Ile Ser Arg Asn Ala Ala Ile Lys Lys Asn Met
        115                 120                 125

Lys Ile Ser Ile Thr Arg Val Asn Ser Gln Ile Thr Lys Glu Asn Gln
    130                 135                 140

Gln Val Asp Phe Pro Thr Glu Val Ile Ser Asp Asp Ser Met Gly Asn
145                 150                 155                 160

Asp Glu Lys Gln Val Ile Gln Gln Gly Gln Ala Gly Glu Lys Glu Val
                165                 170                 175

Phe Thr Lys Ile Val Tyr Glu Asp Gly Lys Ala Val Ser Lys Glu Ile
            180                 185                 190

Val Gly Glu Val Ile Lys Lys Glu Pro Thr Lys Gln Val Phe Lys Val
        195                 200                 205

Gly Thr Leu Gly Val Leu Lys Pro Asp Arg Gly Gly Arg Val Leu Tyr
    210                 215                 220

Lys Lys Ser Leu Gln Val Leu Ala Thr Ala Tyr Thr Asp Asp Phe Ser
225                 230                 235                 240

Phe Gly Ile Thr Ala Ser Gly Thr Lys Val Lys Arg Asp Ser Asp Gly
                245                 250                 255

Tyr Ser Ser Ile Ala Val Asp Pro Thr Val Ile Pro Leu Gly Thr Lys
            260                 265                 270

Leu Tyr Val Pro Gly Tyr Gly Tyr Gly Val Val Ala Glu Asp Thr Gly
        275                 280                 285

Gly Ala Ile Lys Gly Asn Arg Leu Asp Leu Phe Phe Thr Ser Glu Arg
    290                 295                 300

Glu Cys Tyr Asp Trp Gly Ala Lys Asn Val Thr Val Tyr Ile Leu Lys
305                 310                 315                 320
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 81 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Ala Glu Ala Tyr Thr Ala Ser Gly Met His Val Leu Arg Asp Pro Asn
1               5                  10                  15

Gly Tyr Ser Thr Ile Ala Val Asp Pro Ser Val Ile Pro Leu Gly Thr
            20                  25                  30

Lys Leu Tyr Val Glu Gly Tyr Gly Tyr Ala Ile Ile Ala Ala Asp Thr
        35                  40                  45

Gly Gly Ala Ile Lys Gly Asn Arg Val Asp Leu Phe Phe Asn Thr Glu
    50                  55                  60

Ala Glu Ala Ser Asn Trp Gly Val Arg Asn Leu Asp Val Tyr Ile Leu
65                  70                  75                  80

Asn
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Thr Ile Val Val Lys Ser Gly Asp Ser Leu Trp Thr Leu Ala Asn Glu
1               5                  10                  15

Tyr Glu Val Glu Gly Gly Trp Thr Ala Leu Tyr Glu Ala Asn Lys Gly
            20                  25                  30

Ala Val Ser Asp Ala Ala Val Ile Tyr Val Gly Gln Glu Leu Val Leu
        35                  40                  45

Pro Gln Ala
    50
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Thr Ile Lys Val Lys Ser Gly Asp Ser Leu Trp Lys Leu Ser Arg Gln
1               5                  10                  15

Tyr Asp Thr Thr Ile Ser Ala Leu Lys Ser Glu Asn Lys Leu Lys Ser
            20                  25                  30

Thr Val Leu Tyr Val Gly Gln Ser Leu Lys Val Pro Glu Ser
        35                  40                  45
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Thr Ile Lys Val Lys Ser Gly Asp Ser Leu Trp Lys Leu Ala Gln Thr
1               5                  10                  15

Tyr Asn Thr Ser Val Ala Ala Leu Thr Ser Ala Asn His Leu Ser Thr
            20                  25                  30

Thr Val Leu Ser Ile Gly Gln Thr Leu Thr Ile Pro
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Thr Tyr Thr Val Lys Ser Gly Asp Ser Leu Trp Val Ile Ala Gln Lys
1               5                  10                  15

Phe Asn Val Thr Ala Gln Gln Ile Arg Glu Lys Asn Asn Leu Lys Thr
            20                  25                  30

Asp Val Leu Gln Val Gly Gln Lys Leu Val Ile
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Lys Tyr Thr Val Lys Ser Gly Asp Ser Leu Trp Lys Ile Ala Asn Asn
1               5                  10                  15

Ile Asn Leu Thr Val Gln Gln Ile Arg Asn Ile Asn Asn Leu Lys Ser
            20                  25                  30

Asp Val Leu Tyr Val Gly Gln Val Leu Lys Leu
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Thr Tyr Thr Val Lys Ser Gly Asp Thr Ile Trp Ala Leu Ser Ser Lys
1               5                  10                  15

Tyr Gly Thr Ser Val Gln Asn Ile Met Ser Trp Asn Asn Leu Ser Ser
            20                  25                  30

Ser Ser Ile Tyr Val Gly Gln Val Leu Ala Val Lys Gln
        35                  40                  45
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Thr His Ala Val Lys Ser Gly Asp Thr Ile Trp Ala Leu Ser Val Lys
1               5                  10                  15

Tyr Gly Val Ser Val Gln Asp Ile Met Ser Trp Asn Asn Leu Ser Ser
            20                  25                  30

Ser Ser Ile Tyr Val Gly Gln Lys Leu Ala Ile Lys Gln
        35                  40                  45
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Ser Val Lys Val Lys Ser Gly Asp Thr Leu Trp Ala Leu Ser Val Lys
1               5                  10                  15

Tyr Lys Thr Ser Ile Ala Gln Leu Lys Ser Trp Asn His Leu Ser Ser
            20                  25                  30

Asp Thr Ile Tyr Ile Gly Gln Asn Leu Ile Val Ser Gln Ser
        35                  40                  45
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Thr Tyr Thr Val Lys Ser Gly Asp Thr Leu Trp Gly Ile Ser Gln Arg
1               5                  10                  15

Tyr Gly Ile Ser Val Ala Gln Ile Gln Ser Ala Asn Asn Leu Lys Ser
            20                  25                  30

Thr Ile Ile Tyr Ile Gly Gln Lys Leu Leu Leu
        35                  40
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
Thr Tyr Thr Val Lys Lys Gly Asp Thr Leu Trp Asp Ile Ala Gly Arg
1               5                  10                  15

Phe Tyr Gly Asn Ser Thr Gln Trp Arg Lys Ile Trp Asn Ala Asn Lys
            20                  25                  30

Thr Ala Met Ile Lys Arg Ser Lys Arg Asn Ile Arg Gln Pro Gly His
        35                  40                  45

Trp Ile Phe Pro Gly Gln Lys Leu Lys Ile Pro Gln
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Thr Tyr Thr Val Lys Lys Gly Asp Thr Leu Trp Asp Leu Ala Gly Lys
1               5                  10                  15

Phe Tyr Gly Asp Ser Thr Lys Trp Arg Lys Ile Trp Lys Val Asn Lys
            20                  25                  30

Lys Ala Met Ile Lys Arg Ser Lys Arg Asn Ile Arg Gln Pro Gly His
        35                  40                  45
```

Trp Ile Phe Pro Gly Gln Lys Leu Lys Ile Pro Gln
50 55 60

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 167 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Ala Pro Pro Val Glu Leu Ala Ala Asn Asp Leu Pro Ala Pro Leu Gly
1 5 10 15

Glu Pro Leu Pro Ala Ala Pro Ala Asp Pro Ala Pro Pro Ala Asp Leu
20 25 30

Ala Pro Pro Ala Pro Ala Asp Val Ala Pro Pro Val Glu Leu Ala Val
35 40 45

Asn Asp Leu Pro Ala Pro Leu Gly Glu Pro Leu Pro Ala Ala Pro Ala
50 55 60

Asp Pro Ala Pro Pro Ala Asp Leu Ala Pro Pro Ala Pro Ala Asp Leu
65 70 75 80

Ala Pro Pro Ala Pro Ala Asp Leu Ala Pro Pro Ala Pro Ala Asp Leu
85 90 95

Ala Pro Pro Val Glu Leu Ala Val Asn Asp Leu Pro Ala Pro Leu Gly
100 105 110

Glu Pro Leu Pro Ala Ala Pro Ala Glu Leu Ala Pro Pro Ala Asp Leu
115 120 125

Ala Pro Ala Ser Ala Asp Leu Ala Pro Pro Ala Pro Ala Asp Leu Ala
130 135 140

Pro Pro Ala Pro Ala Glu Leu Ala Pro Pro Ala Pro Ala Asp Leu Ala
145 150 155 160

Pro Pro Ala Ala Val Asn Glu
165

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ala Pro Pro Val Glu Leu Ala Ala Asn Asp Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Ala Pro Pro Val Glu Leu Ala Val Asn Asp Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Pro Ala Pro Leu Gly Glu Pro Leu Pro Ala Ala Pro Ala Asp Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Pro Ala Pro Leu Gly Glu Pro Leu Pro Ala Ala Pro Ala Glu Leu
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Pro Ala Pro Pro Ala Asp Leu
1 5

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Ala Pro Pro Ala Pro Ala Asp Leu
1 5

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ala Pro Pro Ala Pro Ala Asp Val
1 5

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Ala Pro Pro Ala Pro Ala Glu Leu
1 5

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Ala Pro Pro Ala Pro Ala Glu Val
1 5

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 478 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```
Met Asn Met Lys Lys Ala Thr Ile Ala Ala Thr Ala Gly Ile Ala Val
1               5                  10                  15

Thr Ala Phe Ala Ala Pro Thr Ile Ala Ser Ala Ser Thr Val Val Val
            20                  25                  30

Glu Ala Gly Asp Thr Leu Trp Gly Ile Ala Gln Ser Lys Gly Thr Thr
        35                  40                  45

Val Asp Ala Ile Lys Lys Ala Asn Asn Leu Thr Thr Asp Lys Ile Val
    50                  55                  60

Pro Gly Gln Lys Leu Gln Val Asn Asn Glu Val Ala Ala Ala Glu Lys
65                  70                  75                  80

Thr Glu Lys Ser Val Ser Ala Thr Trp Leu Asn Val Arg Thr Gly Ala
                85                  90                  95

Gly Val Asp Asn Ser Ile Ile Thr Ser Ile Lys Gly Gly Thr Lys Val
            100                 105                 110

Thr Val Glu Thr Thr Glu Ser Asn Gly Trp His Lys Ile Thr Tyr Asn
        115                 120                 125

Asp Gly Lys Thr Gly Phe Val Asn Gly Lys Tyr Leu Thr Asp Lys Ala
    130                 135                 140

Val Ser Thr Pro Val Ala Pro Thr Gln Glu Val Lys Lys Glu Thr Thr
145                 150                 155                 160

Thr Gln Gln Ala Ala Pro Val Ala Glu Thr Lys Thr Glu Val Lys Gln
                165                 170                 175

Thr Thr Gln Ala Thr Thr Pro Ala Pro Lys Val Ala Glu Thr Lys Glu
            180                 185                 190

Thr Pro Val Ile Asp Gln Asn Ala Thr Thr His Ala Val Lys Ser Gly
        195                 200                 205

Asp Thr Ile Trp Ala Leu Ser Val Lys Tyr Gly Val Ser Val Gln Asp
    210                 215                 220

Ile Met Ser Trp Asn Asn Leu Ser Ser Ser Ser Ile Tyr Val Gly Gln
225                 230                 235                 240

Lys Leu Ala Ile Lys Gln Thr Ala Asn Thr Ala Thr Pro Lys Ala Glu
                245                 250                 255

Val Lys Thr Glu Ala Pro Ala Ala Glu Lys Gln Ala Ala Pro Val Val
            260                 265                 270

Lys Glu Asn Thr Asn Thr Asn Thr Ala Thr Thr Glu Lys Lys Glu Thr
        275                 280                 285

Ala Thr Gln Gln Gln Thr Ala Pro Lys Ala Pro Thr Glu Ala Ala Lys
    290                 295                 300

Pro Ala Pro Ala Pro Ser Thr Asn Thr Asn Ala Asn Lys Thr Asn Thr
305                 310                 315                 320
```

```
Asn Thr Asn Thr Asn Asn Thr Asn Thr Pro Ser Lys Asn Thr Asn Thr
                325                 330                 335

Asn Ser Asn Thr Asn Thr Asn Thr Asn Ser Asn Thr Asn Ala Asn Gln
            340                 345                 350

Gly Ser Ser Asn Asn Asn Ser Asn Ser Ser Ala Ser Ala Ile Ile Ala
        355                 360                 365

Glu Ala Gln Lys His Leu Gly Lys Ala Tyr Ser Trp Gly Gly Asn Gly
    370                 375                 380

Pro Thr Thr Phe Asp Cys Ser Gly Tyr Thr Lys Tyr Val Phe Ala Lys
385                 390                 395                 400

Ala Gly Ile Ser Leu Pro Arg Thr Ser Gly Ala Gln Tyr Ala Ser Thr
                405                 410                 415

Thr Arg Ile Ser Glu Ser Gln Ala Lys Pro Gly Asp Leu Val Phe Phe
            420                 425                 430

Asp Tyr Gly Ser Gly Ile Ser His Val Gly Ile Tyr Val Gly Asn Gly
        435                 440                 445

Gln Met Ile Asn Ala Gln Asp Asn Gly Val Lys Tyr Asp Asn Ile His
    450                 455                 460

Gly Ser Gly Trp Gly Lys Tyr Leu Val Gly Phe Gly Arg Val
465                 470                 475
```

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 758 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 66..728

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

```
ACCAAGGAGA AGGACGACCC CGGTGTGCCT CGGCCGCCGA TCAGCGAGGA CTCGCCATGG       60

ACACC ATG ACT CTC TTC ACC ACT TCC GCC ACC CGC TCC CGC CGT GCC           107
      Met Thr Leu Phe Thr Thr Ser Ala Thr Arg Ser Arg Arg Ala
        1               5                  10

ACC GCC TCG ATC GTC GCG GGC ATG ACC CTC GCC GGC GCC GCC GCC GTG        155
Thr Ala Ser Ile Val Ala Gly Met Thr Leu Ala Gly Ala Ala Ala Val
 15                 20                  25                  30

GGC TTC TCC GCC CCG GCC CAG GCC GCC ACC GTG GAC ACC TGG GAC CGC        203
Gly Phe Ser Ala Pro Ala Gln Ala Ala Thr Val Asp Thr Trp Asp Arg
                35                  40                  45

CTC GCC GAG TGC GAG TCC AAC GGC ACC TGG GAC ATC AAC ACC GGC AAC        251
```

```
Leu Ala Glu Cys Glu Ser Asn Gly Thr Trp Asp Ile Asn Thr Gly Asn
                50                  55                  60

GGC TTC TAC GGC GGC GTG CAG TTC ACC CTG TCC TCC TGG CAG GCC GTC       299
Gly Phe Tyr Gly Gly Val Gln Phe Thr Leu Ser Ser Trp Gln Ala Val
        65                  70                  75

GGC GGC GAA GGC TAC CCG CAC CAG GCC TCG AAG GCC GAG CAG ATC AAG       347
Gly Gly Glu Gly Tyr Pro His Gln Ala Ser Lys Ala Glu Gln Ile Lys
     80                  85                  90

CGC GCC GAG ATC CTC CAG GAC CTG CAG GGC TGG GGC GCG TGG CCG CTG       395
Arg Ala Glu Ile Leu Gln Asp Leu Gln Gly Trp Gly Ala Trp Pro Leu
 95                 100                 105                 110

TGC TCG CAG AAG CTG GGC CTG ACC CAG GCT GAC GCG GAC GCC GGT GAC       443
Cys Ser Gln Lys Leu Gly Leu Thr Gln Ala Asp Ala Asp Ala Gly Asp
                115                 120                 125

GTG GAC GCC ACC GAG GCC GCC CCG GTC GCC GTG GAG CGC ACG GCC ACC       491
Val Asp Ala Thr Glu Ala Ala Pro Val Ala Val Glu Arg Thr Ala Thr
            130                 135                 140

GTG CAG CGC CAG TCC GCC GCG GAC GAG GCT GCC GCC GAG CAG GCC GCT       539
Val Gln Arg Gln Ser Ala Ala Asp Glu Ala Ala Ala Glu Gln Ala Ala
        145                 150                 155

GCC GCG GAG CAG GCC GTC GTC GCC GAG GCC GAG ACC ATC GTC GTC AAG       587
Ala Ala Glu Gln Ala Val Val Ala Glu Ala Glu Thr Ile Val Val Lys
    160                 165                 170

TCC GGT GAC TCC CTC TGG ACG CTC GCC AAC GAG TAC GAG GTG GAG GGT       635
Ser Gly Asp Ser Leu Trp Thr Leu Ala Asn Glu Tyr Glu Val Glu Gly
175                 180                 185                 190

GGC TGG ACC GCC CTC TAC GAG GCC AAC AAG GGC GCC GTC TCC GAC GCC       683
Gly Trp Thr Ala Leu Tyr Glu Ala Asn Lys Gly Ala Val Ser Asp Ala
                195                 200                 205

GCC GTG ATC TAC GTC GGC CAG GAG CTC GTC CTG CCG CAG GCC TGAGACGCCT    735
Ala Val Ile Tyr Val Gly Gln Glu Leu Val Leu Pro Gln Ala
            210                 215                 220

GACCGGCCCC CCGGACCGGT ACC                                             758
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 220 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
Met Thr Leu Phe Thr Thr Ser Ala Thr Arg Ser Arg Arg Ala Thr Ala
  1               5                  10                  15

Ser Ile Val Ala Gly Met Thr Leu Ala Gly Ala Ala Ala Val Gly Phe
```

```
                20                  25                  30

Ser Ala Pro Ala Gln Ala Ala Thr Val Asp Thr Trp Asp Arg Leu Ala
            35                  40                  45

Glu Cys Glu Ser Asn Gly Thr Trp Asp Ile Asn Thr Gly Asn Gly Phe
        50                  55                  60

Tyr Gly Gly Val Gln Phe Thr Leu Ser Ser Trp Gln Ala Val Gly Gly
 65                  70                  75                  80

Glu Gly Tyr Pro His Gln Ala Ser Lys Ala Glu Gln Ile Lys Arg Ala
                85                  90                  95

Glu Ile Leu Gln Asp Leu Gln Gly Trp Gly Ala Trp Pro Leu Cys Ser
            100                 105                 110

Gln Lys Leu Gly Leu Thr Gln Ala Asp Ala Asp Ala Gly Asp Val Asp
        115                 120                 125

Ala Thr Glu Ala Ala Pro Val Ala Val Glu Arg Thr Ala Thr Val Gln
    130                 135                 140

Arg Gln Ser Ala Ala Asp Glu Ala Ala Ala Glu Gln Ala Ala Ala Ala
145                 150                 155                 160

Glu Gln Ala Val Val Ala Glu Ala Glu Thr Ile Val Val Lys Ser Gly
                165                 170                 175

Asp Ser Leu Trp Thr Leu Ala Asn Glu Tyr Glu Val Glu Gly Gly Trp
            180                 185                 190

Thr Ala Leu Tyr Glu Ala Asn Lys Gly Ala Val Ser Asp Ala Ala Val
        195                 200                 205

Ile Tyr Val Gly Gln Glu Leu Val Leu Pro Gln Ala
    210                 215                 220
```

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCSACSGTSG ACACSTGGGA CCGSCTSGCS GAG 33

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Ala Thr Val Asp Thr Trp Asp Arg Leu Ala Glu Glu Xaa Ser Asn Gly
1 5 10 15

Thr Xaa Asp

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CCGCCGTAGA AGCCGTTG 18

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

AGTTCACCCT GTCCTCCTG 19

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 9
(D) OTHER INFORMATION: /note= "N is inosine"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 15
(D) OTHER INFORMATION: /note= "N is inosine"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 21
(D) OTHER INFORMATION: /note= "N is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GCYTGRTGNG GRTANCCYTC NCC 23

(2) INFORMATION FOR SEQ ID NO:42:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Val Gly Gly Glu Gly Tyr Pro His Gln Ala Ser Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 182 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Ala Thr Val Asp Thr Trp Asp Arg Leu Ala Glu Cys Glu Ser Asn Gly
1 5 10 15

Thr Trp Asp Ile Asn Thr Gly Asn Gly Phe Tyr Gly Gly Val Gln Phe
20 25 30

Thr Leu Ser Ser Trp Gln Ala Val Gly Gly Glu Gly Tyr Pro His Gln
35 40 45

Ala Ser Lys Ala Glu Gln Ile Lys Arg Ala Glu Ile Leu Gln Asp Leu
50 55 60

Gln Gly Trp Gly Ala Trp Pro Leu Cys Ser Gln Lys Leu Gly Leu Thr
65 70 75 80

Gln Ala Asp Ala Asp Ala Gly Asp Val Asp Ala Thr Glu Ala Ala Pro
85 90 95

Val Ala Val Glu Arg Thr Ala Thr Val Gln Arg Gln Ser Ala Ala Asp
100 105 110

Glu Ala Ala Ala Glu Gln Ala Ala Ala Ala Glu Gln Ala Val Val Ala
115 120 125

Glu Ala Glu Thr Ile Val Val Lys Ser Gly Asp Ser Leu Trp Thr Leu
130 135 140

Ala Asn Glu Tyr Glu Val Glu Gly Gly Trp Thr Ala Leu Tyr Glu Ala
145 150 155 160

Asn Lys Gly Ala Val Ser Asp Ala Ala Val Ile Tyr Val Gly Gln Glu

```
                    165                 170                 175

     Leu Val Leu Pro Gln Ala
                 180
```

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 299 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..299

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

```
GG ATC CGC ACC GCC GCG GTA ACC CTG GTC GCC GCG ACC GCA CTC GGG          47
   Ile Arg Thr Ala Ala Val Thr Leu Val Ala Ala Thr Ala Leu Gly
    1               5                  10                  15

GCG ACC GGC GAA GCG GTG GCC GCG CCC TCG GCG CCC CTG CGC ACC GAC         95
Ala Thr Gly Glu Ala Val Ala Ala Pro Ser Ala Pro Leu Arg Thr Asp
                20                  25                  30

TGG GAC GCC ATC GCC GCG TGC GAG TCC AGC GGC AAC TGG CAG GCG AAC        143
Trp Asp Ala Ile Ala Ala Cys Glu Ser Ser Gly Asn Trp Gln Ala Asn
            35                  40                  45

ACC GGC AAC GGC TAC TAC GGC GGC CTG CAG TTC GCA CGG TCC AGC TGG        191
Thr Gly Asn Gly Tyr Tyr Gly Gly Leu Gln Phe Ala Arg Ser Ser Trp
        50                  55                  60

ATC GCC GCC GGC GGC CTC AAG TAC GCC CCG CGC GCG GAC CTC GCC ACC        239
Ile Ala Ala Gly Gly Leu Lys Tyr Ala Pro Arg Ala Asp Leu Ala Thr
    65                  70                  75

CGC GGC GAG CAG ATC GCC GTG GCG GAA CGC CTC GCC CGT CTG CAG GGG        287
Arg Gly Glu Gln Ile Ala Val Ala Glu Arg Leu Ala Arg Leu Gln Gly
80                  85                  90                  95

ATG TCC GCC TGG                                                        299
Met Ser Ala Trp
```

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Ile Arg Thr Ala Ala Val Thr Leu Val Ala Ala Thr Ala Leu Gly Ala
1 5 10 15

Thr Gly Glu Ala Val Ala Ala Pro Ser Ala Pro Leu Arg Thr Asp Trp
20 25 30

Asp Ala Ile Ala Ala Cys Glu Ser Ser Gly Asn Trp Gln Ala Asn Thr
35 40 45

Gly Asn Gly Tyr Tyr Gly Gly Leu Gln Phe Ala Arg Ser Ser Trp Ile
50 55 60

Ala Ala Gly Gly Leu Lys Tyr Ala Pro Arg Ala Asp Leu Ala Thr Arg
65 70 75 80

Gly Glu Gln Ile Ala Val Ala Glu Arg Leu Ala Arg Leu Gln Gly Met
85 90 95

Ser Ala Trp

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GTCAGAATTC ATATGGCCAC CGTGGACACC TGGG 34

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TGACGGATCC TATTAGGCCT GCGGCAGGAC GAG 33

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

ATCAGAATTC ATATGGACGA CATCGATTGG GACGC 35

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

CGCAGGATCC CCTCAATCGT CCCTGCTCC 29

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

GAAGAGAATT CCTTCCATCA CGA 23

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

CCAAACGAAT TCGGTCAATC AC 22

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

GCAAGGATCC CAGACTAAAA AAACAG 26

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

ATCAGGATCC ATATTATTAG TTTAAGA 27

2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 663 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single stranded
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..663

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

```
atg act ctc ttc acc act tcc gcc acc cgc tcc cgc cgt gcc acc gcc   48
Met Thr Leu Phe Thr Thr Ser Ala Thr Arg Ser Arg Arg Ala Thr Ala
  1               5                  10                  15

tcg atc gtc gcg ggc atg acc ctc gcc ggc gcc gcc gcc gtg ggc ttc   96
Ser Ile Val Ala Gly Met Thr Leu Ala Gly Ala Ala Ala Val Gly Phe
            20                  25                  30

tcc gcc ccg gcc cag gcc gcc acc gtg gac acc tgg gac cgc ctc gcc   144
Ser Ala Pro Ala Gln Ala Ala Thr Val Asp Thr Trp Asp Arg Leu Ala
        35                  40                  45

gag tgc gag tcc aac ggc acc tgg gac atc aac acc ggc aac ggc ttc   192
Glu Cys Glu Ser Asn Gly Thr Trp Asp Ile Asn Thr Gly Asn Gly Phe
    50                  55                  60

tac ggc ggc gtg cag ttc acc ctg tcc tcc tgg cag gcc gtc ggc ggc   240
Tyr Gly Gly Val Gln Phe Thr Leu Ser Ser Trp Gln Ala Val Gly Gly
65                  70                  75                  80

gaa ggc tac ccg cac cag gcc tcg aag gcc gag cag atc aag cgc gcc   288
Glu Gly Tyr Pro His Gln Ala Ser Lys Ala Glu Gln Ile Lys Arg Ala
                85                  90                  95

gag atc ctc cag gac ctg cag ggc tgg ggc gcg tgg ccg ctg tgc tcg   336
Glu Ile Leu Gln Asp Leu Gln Gly Trp Gly Ala Trp Pro Leu Cys Ser
            100                 105                 110

cag aag ctg ggc ctg acc cag gct gac gcg gac gcc ggt gac gtg gac   384
Gln Lys Leu Gly Leu Thr Gln Ala Asp Ala Asp Ala Gly Asp Val Asp
        115                 120                 125

gcc acc gag gcc gcc ccg gtc gcc gtg gag cgc acg gcc acc gtg cag   432
```

```
Ala Thr Glu Ala Ala Pro Val Ala Val Glu Arg Thr Ala Thr Val Gln
    130                 135                 140

cgc cag tcc gcc gcg gac gag gct gcc gcc gag cag gcc gct gcc gcg   480
Arg Gln Ser Ala Ala Asp Glu Ala Ala Ala Glu Gln Ala Ala Ala Ala
145                 150                 155                 160

gag cag gcc gtc gtc gcc gag gcc gag acc atc gtc gtc aag tcc ggt   528
Glu Gln Ala Val Val Ala Glu Ala Glu Thr Ile Val Val Lys Ser Gly
                165                 170                 175

gac tcc ctc tgg acg ctc gcc aac gag tac gag gtg gag ggt ggc tgg   576
Asp Ser Leu Trp Thr Leu Ala Asn Glu Tyr Glu Val Glu Gly Gly Trp
            180                 185                 190

acc gcc ctc tac gag gcc aac aag ggc gcc gtc tcc gac gcc gcc gtg   624
Thr Ala Leu Tyr Glu Ala Asn Lys Gly Ala Val Ser Asp Ala Ala Val
        195                 200                 205

atc tac gtc ggc cag gag ctc gtc ctg ccg cag gcc tga               663
Ile Tyr Val Gly Gln Glu Leu Val Leu Pro Gln Ala
    210                 215                 220
```

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

```
Ala Pro Pro Ala Asp Leu
1               5
```

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

```
Ala Pro Ala Ser Ala Asp Leu
1               5
```

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Ala Pro Pro Ala Pro Ala Glu Leu
1 5

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

Ala Pro Pro Ala
1

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Ala Val Asn Asp
1

FIG. 1A

```
SEQ ID NO: 1  MtubZ94752   mlrlvvgalllvlafaggyavaacktvtltvdgtamrvtmksrvidive   50
              MtubZ94752   engfsvddrddlypaagvqvhdadtivlrrsrplqislddhdcbgvwtta  100
              MtubZ94752   stvdealaqlamtdtapaaasrasrvplsgmalpvvsaktvqlndgglvr   ...
              MtubZ94752   tvhlpapnvaglllsaagvpllqsdhvvpaatapivegmqiqvtrnrikkv  200
SEQ ID NO: 2  MtubMTV008   ----------------mpvgwlwrartakgttlknarttliaaaiagt   32

SEQ ID NO: 3  Mlepl04666   ------------------------------------msesyrkl        8
SEQ ID NO: 4  MtubMTV043   ------------------------------------msgrhrkpt       9
              MtubZ94752   terlplppnarrvedpemnmsrevvedpgvpgtqdvtfavaevngvetgr  250
SEQ ID NO: 36 Mlutz96935   ------------------------------------mtlfttsat       9
SEQ ID NO: 5  MlepL01095   ------------------------------------mpgemldvrklc    12
SEQ ID NO: 6  MtubU38939   ----------mhplpadhqrsrcnrhpisplslignisatsqdmssmt    38
SEQ ID NO: 7  MtubZ81368   ------------------------------mtpglltaqagrprdrca    19
              MtubMTV008   lvttspagianaddaglдpnaaagpdavgfdpnlppapdaapvdtppape  82

SEQ ID NO: 8  Scoeli6C12$  ---irtaavtlvaatalgatgeavaapsaplrtDWDAIAACESSGNWQAN  25
              Mlepl04666   ttssiivakitftgamlddgsialggaspatdsEWDQVARCESGGNWSIN  58
              MtubMTV043   tsnvsvakiaftgavlggggiamaaqataatdgEWDQVARCESGGNWSIN  59
              MtubZ94752   lpvanvvtpaheavvrvgtkpgtevppvidgsIWDAIAGCEAGGNWAIN  300
              Mlutz96935   rsrratasivaqmtlagaaavqfsapaqaatvdTWDRLAECESNGTWDIN  59
              MlepL01095   klfvksavvsgivtasmalststqmanavprePNWDAVAQCESGRNWRAN  62
              MtubU38939   riakpliksamaaglvtasmslstavahagpsPNWDAVAQCESGGNWAAN  88
              MtubZ81368   rivctvfietavvatmfvallglstisskaddIDWDAIAQCESGGNWAAN  69
              MtubMTV008   dagfdpnlppplapdflsppaeeappvpvaysVNWDAIAQCESGGNWSIN  132
                                                          ***.* *******  *

              Scoeli6C12$  TGNGYYGGLQFARSSWIAAGGLKYAPRADLATRGEQIAVAERLARLQGMS  75
              Mlepl04666   TGNGYLGGLQFSQGTWASHGGGEYAPSAQLATREQQIAVAERVLATQGSG  108
              MtubMTV043   TGNGYLGGLQFTQSTWAAHGGGEFAPSAQLASREQQIAVGERVLATQGRG  109
              MtubZ94752   TGNGYYGGVQFDQGTWEANGGLRYAPRADLATREEQIAVAEVTRLRQGWG  350
              Mlutz96935   TGNGFYGGVQFTLSSWQAVGGEG---YPHQASKAEQIKRAEILQDLQGWG  106
              MlepL01095   TGNGFYGGLQFKPTIWARYGGVG---NPAGASREQQITVANRVLADQGLD  109
              MtubU38939   TGNGKYGGLQFKPATWAAFGGVG---NPAAASREQQIAVANRVLAEQGLD  135
              MtubZ81368   TGNGLYGGLQISQATWDSNGGVG---SPAAASPQQQIEVADNIMKTQGPG  116
              MtubMTV008   TGNGYYGGLQFTAGTWRANGGSG---SAANASREEQIRVAENVLRSQGIR  179
                           ****.******   .* . **          *.***** *** ..  **

              Scoeli6C12$  AW-------------------------------------------------  78
              Mlepl04666   AWPACGHGLSGPSLQEVLPAG---MGAPW-----INGAPAPLAPPPPAEPAP  152
              MtubMTV043   AWPVCGRGLSNATPREVLPASaaMDAPldaaaVNGEPAPLA-PPPADPAP  158
              MtubZ94752   AWPVCAaragar-----------------------------------------  362
              Mlutz96935   AWPLCSQKLgltqadadagdvdateaapvavertatvqrqsaadeaaaeq  156
              MlepL01095   AWPKCGAASDLPITLWSHPAQGVKQIINDIIqmgdttlaaialngl-----  155
              MtubU38939   AWPTCGAASGLPIALWSKPAQGIKQIINEIIwagiqasipr----------  176
              MtubZ81368   AWPKCSscsqgdaplgslthiltflaaetggcsgsrdd-------------  154
              MtubMTV008   AWPVCGrrg-------------------------------------------  188
                           *** *.
```

FIG. 1A (CONT.)

```
Mlepl04666     pqppadnf-------------------------PPTPGDVPSPLarp-----   174
MtubMTV043     pvelaandlpaplgeplpaapadpappadlaPPAPADVAPPVelavndlp    208
MlutZ96935     aaaaeqavvaeaetivvksgdslwtlaneyeveggwtalyeankgavsda    206

MtubMTV043     aplgeplpaapadpappadlappapadlappapadlappapadlappvel   258
MlutZ96935     aviyvgqelvlpqa-----------------------------------   220

MtubMTV043     avndlpaplgeplpaapaelappadlapasadlappapadlappapaela   308

MtubMTV043     ppapadlappaavneqtapgdqpatapggpvglatdlelpepdpqpadap   358

MtubMTV043     ppgdvteapaetpqvsniaytkklwqairaqdvcgndaldslaqpyvig-   407
```

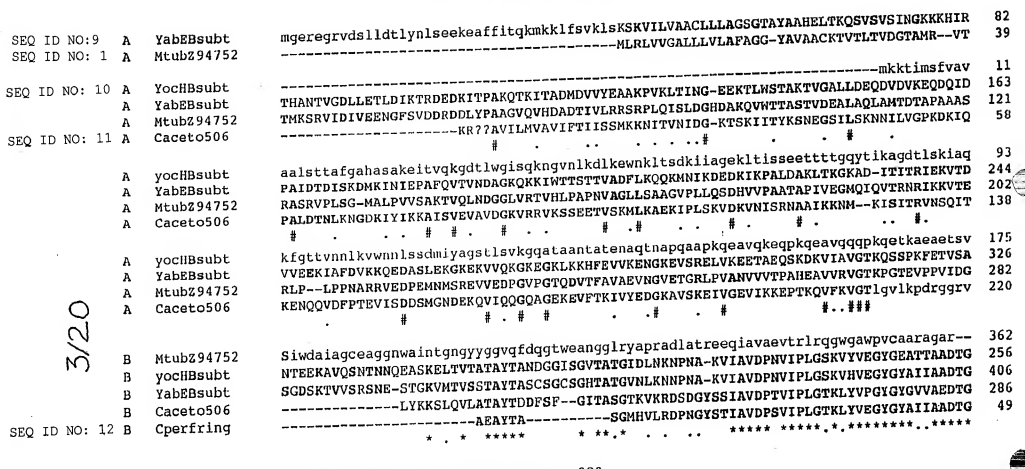

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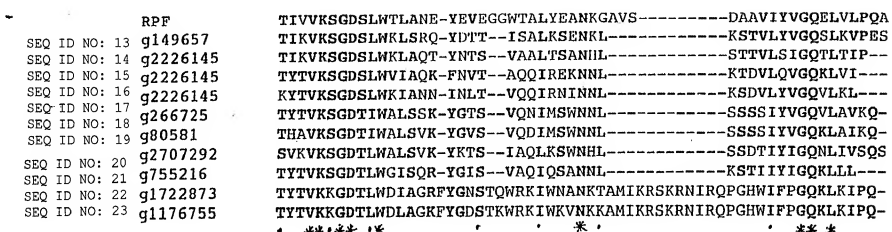
FIG. 1B



4/20

FIG. 1C

09/445289

SEQ ID NO: 4 1 msgrhrkpttsnvsvakiaftgavlggggiamaaqataatdgewdqvarcesggnwsintgngylgg
lqftqstwaahgggefapsaqlasreqqiavgervlatqgrgawpvcgrglsnatprevlpasaamd
apldaaavngepaplapppadp 156

157 appvelaandlpaplgeplpaapadpappadlappapadv 196
197 appvelavndlpaplgeplpaapadpappadlappapadlappapadl 252
253 appvelavndlpaplgeplpaapaelappadlap-asadlappapadlappapaelappapadlappa
320 ------avne 323

324 qtapgdqpatapggpvglatdlelpepdpqpadapppgdvteapaetpqvsniaytkklwqaira
389 qdvcgndaldslaqpyvig* 407

Motif	sequence		
A	157 appvelaandl	167	SEQ ID NO: 25
B'	168 paplgeplpaapad	181	SEQ ID NO: 28
C	182 pappadl	188	SEQ ID NO: 29
D	189 appapadv	196	SEQ ID NO: 31
A	197 appvelavndl	207	SEQ ID NO: 26
B'	208 paplgeplpaapad	221	SEQ ID NO: 28
C	222 pappadl	228	SEQ ID NO: 29
D	229 appapadl	236	SEQ ID NO: 30
D	237 appapadl	244	SEQ ID NO: 30
D	245 appapadl	252	SEQ ID NO: 30
A	253 appvelavndl	263	SEQ ID NO: 26
B	264 paplgeplpaapael	278	SEQ ID NO: 27
'C	279 appadl	284	SEQ ID NO:55
D*	285 apasadl	291	SEQ ID NO: 56
D	292 appapadl	299	SEQ ID NO: 30
D	300 appapael	307	SEQ ID NO:57
D	308 appapadl	315	SEQ ID NO: 30
D'	316 appa	319	SEQ ID NO: 58
'A'	320 avne	323	SEQ ID NO: 59

A = appvela[av]ndl

B = paplgeplpaapa[de]l

C = pappadl

D = appapa[de][lv]

Fig. 1D

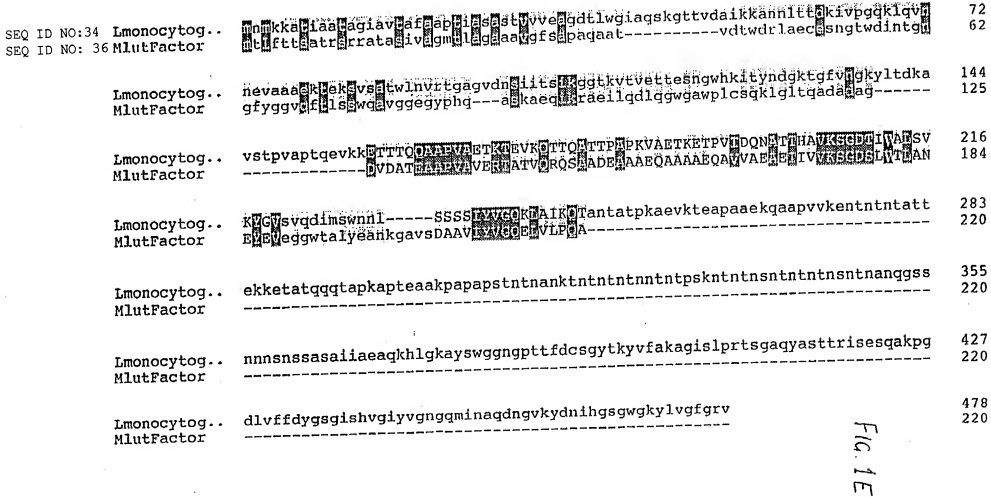

SEQ ID NO:34 Lmonocytog..
SEQ ID NO: 36 MlutFactor
72
62
Lmonocytog..
MlutFactor
144
125
Lmonocytog..
MlutFactor
216
184
Lmonocytog..
MlutFactor
283
220
Lmonocytog..
MlutFactor
355
220
Lmonocytog..
MlutFactor
427
220
Lmonocytog..
MlutFactor
478
220

FIG. 1E

SEQ ID NO:35 1 accaaggagaaggacgaccccggtgtgcctcggccgccgatcagcgaggactcgccatgg 60

61 acaccatgactctcttcaccacttccgccacccgctcccgccgtgccaccgcctcgatcg 120
M T L F T T S A T R S R R A T A S I V

SEQ ID NO: 37 g

121 tcgcgggcatgaccctcgccggcgccgccgccgtgggcttctccgccccggccaggccg 180
A G M T L A G A A A V G F S A P A Q A A

SEQ ID NO: 38 *A*

oligo A1>>>

csacsgtsgacacstgggaccgsctsgcsgag

181 ccaccgtggacacctgggaccgctctgccgagtgcgagtccaacggcacctgggacatca 240
T V D T W D R L A E C E S N G T W D I N
T V D T W D R L A E E x S N G T x D

<<< oligo G2 SEQ ID NO: 40 oligo G1>>>

SEQ ID NO: 39 gttgccgaagatgccgcc agttcaccctgtcctcctg

241 acaccggcaacggcttctacggcggcgtgcagttcaccctgtcctcctggcaggccgtcg 300
T G N G F Y G G V Q F T L S S W Q A V G

SEQ ID NO: 42 *G*

<<< oligo A2

SEQ ID NO: 41 ccictycciatrggigtrgtycg

301 gcggcgaaggctacccgcaccaggcctcgaaggccgagcagatcaagcgcgccgagatcc 360
G E G Y P H Q A S K A E Q I K R A E I L
G E G Y P H Q A S K

361 tccaggacctgcagggctggggcgcgtggccgctgtgctcgcagaagctgggcctgaccc 420
Q D L Q G W G A W P L C S Q K L G L T Q

421 aggctgacgcggacgccggtgacgtggacgccaccgaggccgccccggtcgccgtggagc 480
A D A D A G D V D A T E A A P V A V E R

481 gcacggccaccgtgcagcgccagtccgccgcggacgaggctgccgccgagcaggccgctg 540
T A T V Q R Q S A A D E A A A E Q A A A

541 ccgcggagcaggccgtcgtcgccgaggccgagaccatcgtcgtcaagtccggtgactccc 600
A E Q A V V A E A E T I V V K S G D S L

601 tctggacgctcgccaacgagtacgaggtggagggtggctggaccgccctctacgaggcca 660
W T L A N E Y E V E G G W T A L Y E A N

661 acaagggcgccgtctccgacgccgccgtgatctacgtcggccaggagctcgtcctgccgc 720
K G A V S D A A V I Y V G Q E L V L P Q

721 aggcctgagacgcctgaccggccccccggaccggtacc 758
A *

SEQ ID NO: 43 1 ATVDTWDRLA ECESNGTWDI NTGNGFYGGV QFTLSSWQAV GGEGYPHQAS KAEQIKRAEI 60
61 LQDLQGWGAW PLCSQKLGLT QADADAGDVD ATEAAPVAVE RTATVQRQSA ADEAAAEQAA 120
121 AAEQAVVAEA ETIVVKSGDS LWTLANEYEV EGGWTALYEA NKGAVSDAAV IYVGQELVLP QA 182

FIG. 2A

```
SEQ ID NO:44  ggatccgcaccgccgcggtaaccctggtcgccgcgaccgcactcggggcgaccggcgaag  60
SEQ ID NO: 45   I  R  T  A  A  V  T  L  V  A  A  T  A  L  G  A  T  G  E  A

              cggtggccgcgccctcggcgcccctgcgcaccgactgggacgccatcgccgcgtgcgagt 120
                V  A  A  P  S  A  P  L  R  T  D  W  D  A  I  A  A  C  E  S

              ccagcggcaactggcaggcgaacaccggcaacggctactacggcggcctgcagttcgcac 180
                S  G  N  W  Q  A  N  T  G  N  G  Y  Y  G  G  L  Q  F  A  R

              ggtccagctggatcgccgccggcggcctcaagtacgccccgcgcgcggacctcgccaccc 240
                S  S  W  I  A  A  G  G  L  K  Y  A  P  R  A  D  L  A  T  R

              gcggcgagcagatcgccgtggcggaacgcctcgcccgtctgcaggggatgtccgcctgg  299
                G  E  Q  I  A  V  A  E  R  L  A  R  L  Q  G  M  S  A  W
```

FIG. 2B

FIG. 3

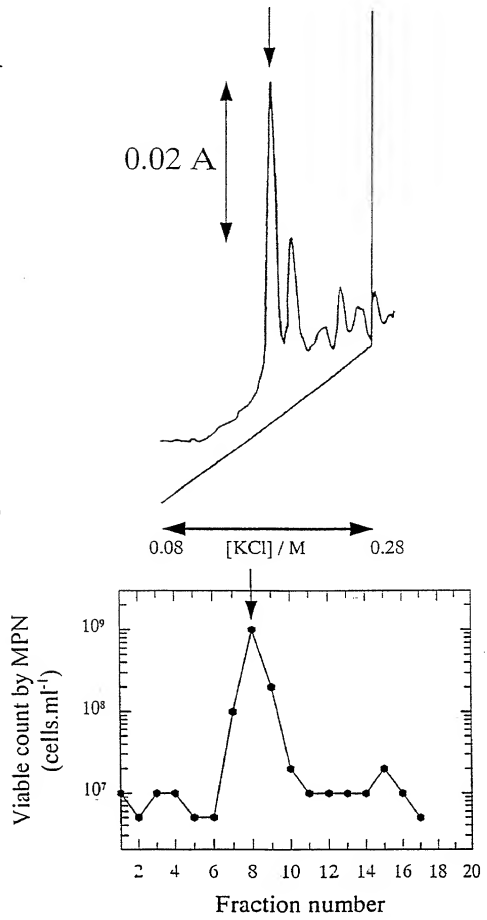
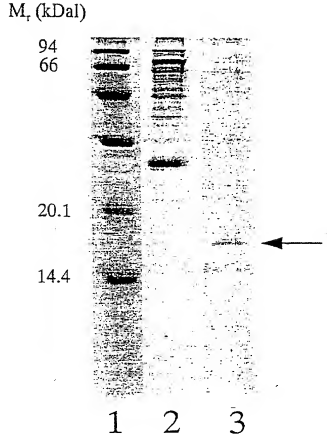

A
0.02 A
B
0.08
[KCl] / M
0.28
Viable count by MPN
(cells.ml-1)
10⁹
10⁸
10⁷
2
4
6
8
10
12
14
16
18
20
Fraction number

FIG. 3

C.


M, (kDal)
94
66
20.1
14.4
1
2
3

D.

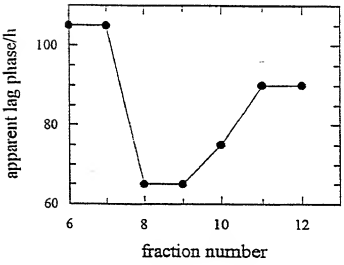
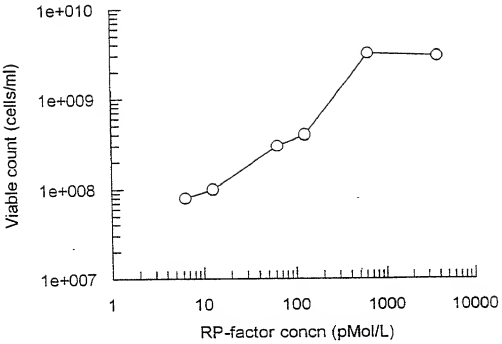

apparent lag phase/h
100
80
60
6
8
10
12
fraction number

FIG. 4

A


1e+010
1e+009
1e+008
1e+007
Viable count (cells/ml)
1
10
100
1000
10000
RP-factor concn (pMol/L)

B

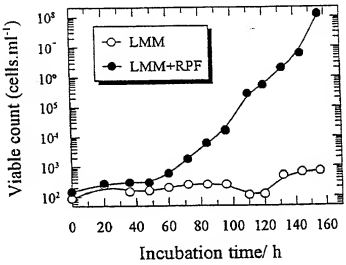
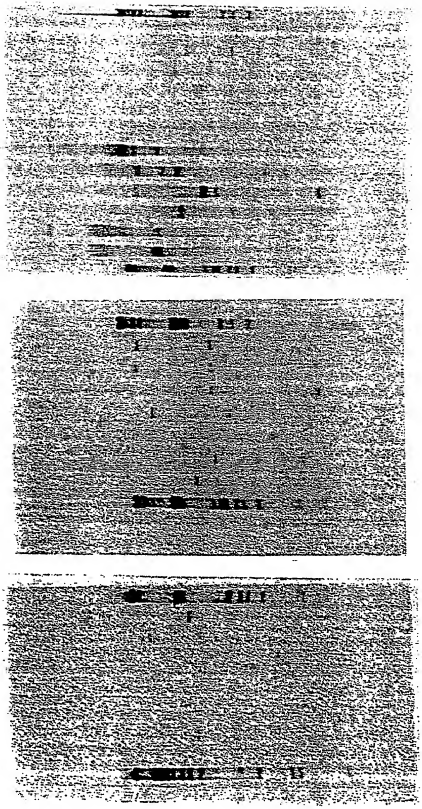

LMM
LMM+RPF
Viable count (cells.ml-1)
0 20 40 60 80 100 120 140 160
Incubation time/ h

FIG.5



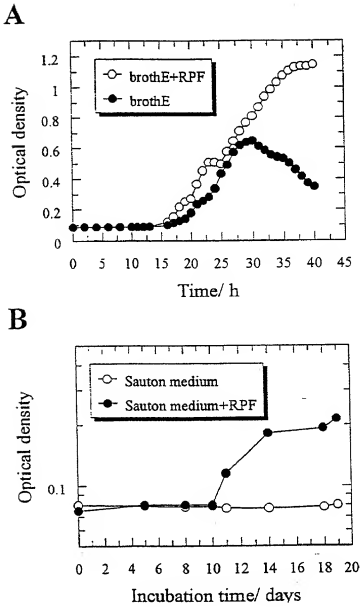
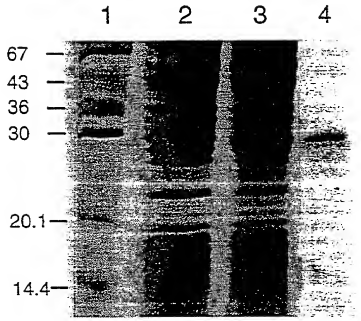

A
brothE+RPF
brothE
Optical density
1.2
1
0.8
0.6
0.4
0.2
0
0 5 10 15 20 25 30 35 40 45
Time/ h
B
Sauton medium
Sauton medium+RPF
Optical density
0.1
0 2 4 6 8 10 12 14 16 18 20
Incubation time/ days

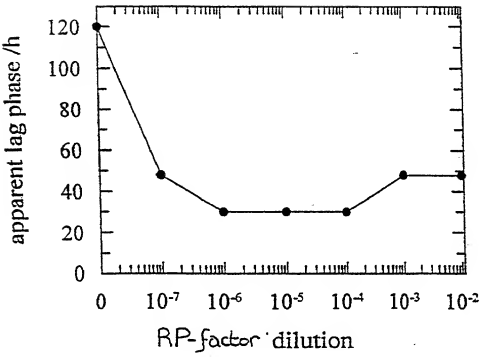
FIG. 6

FIG. 7

A


1
2
3
4
67
43
36
30
20.1
14.4

B


apparent lag phase /h
120
100
80
60
40
20
0
0
10-7
10-6
10-5
10-4
10-3
10-2
RP-factor dilution

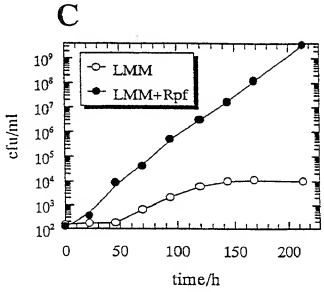


FIG. 7

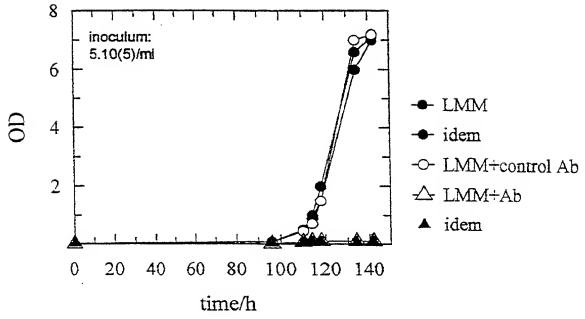

inoculum:
5.10(5)/ml
OD
8
6
4
2
0
20
40
60
80
100
120
140
time/h
LMM
idem
LMM+control Ab
LMM+Ab
idem

FIG. 8A

FIG. 8 B

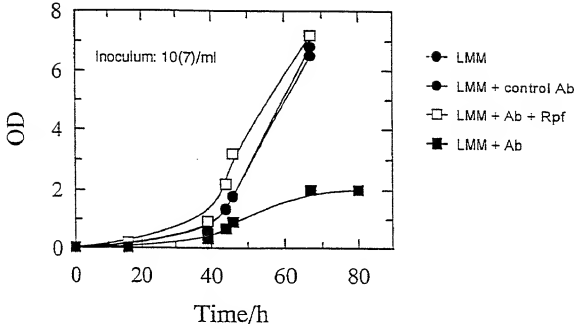

Inoculum: 10(7)/ml
OD
Time/h
0
2
4
6
8
0
20
40
60
80
LMM
LMM + control Ab
LMM + Ab + Rpf
LMM + Ab

FIG.9A

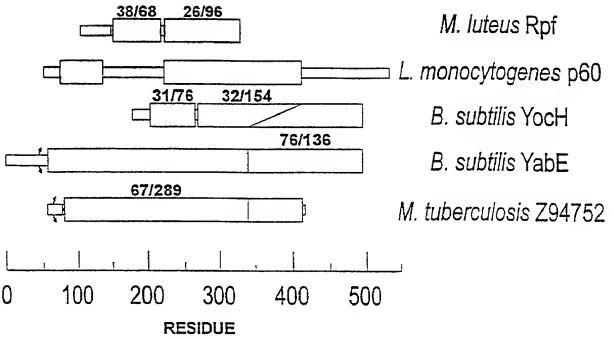

38/68
26/96
M. luteus Rpf
L. monocytogenes p60
31/76
32/154
B. subtilis YocH
76/136
B. subtilis YabE
67/289
M. tuberculosis Z94752
0
100
200
300
400
500
RESIDUE

FIG. 9B

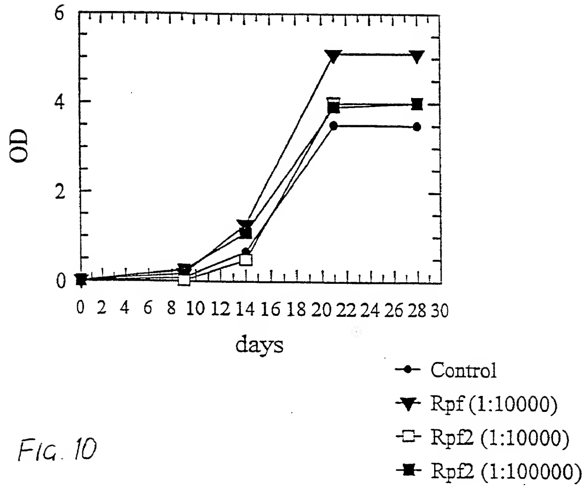


FIG. 10

BACTERIAL PHEROMONES AND USES THEREFOR

Field of the invention

The present invention relates to RP-factors, their cognate receptors, convertases, respective genes and to inhibitors or mimetics thereof. In particular, the invention relates to antibodies, pharmaceutical compositions and (therapeutic, diagnostic) methods based on the RP-factors and their receptors/convertases.

Introduction

Bacterial pheromones

It is known that certain chemicals may mediate intercellular communication in bacterial cultures. Such communication has been shown to be of importance during sporulation, conjugation, changes in virulence and in bioluminescence. It is now clear that a variety of different autocrine and/or paracrine chemical compounds ("pheromones") produced as secondary metabolites are responsible for such social behaviour in prokaryotes (see e.g. Kell et al, 1995, Trends Ecol. Evolution, 10, 126-129).

Pheromones may be distinguished from nutrients inter alia in that: (i) they are produced by the organisms themselves, (ii) they are active at very low concentrations (e.g. at picomolar or nanomolar concentrations), and (iii) with the exception of prohormone processing, their *metabolism* is not necessary for activity (although they may of course ultimately be degraded).

The chemical nature of these pheromonal compounds varies widely: those associated with Gram-negative organisms tend to be of low molecular weight (e.g. N-acyl homoserine lactone derivatives), whilst a number of Gram-positive organisms use proteins and polypeptides (Kell et al,1995, *ibidem*).

Pheromones are also known to play an important role in the development of bacterial cultures. For unstressed (uninjured) bacteria and optimal growth media, the "self-promoting" mode of culture growth is normally masked due to the high rate of production of growth factors and the sensitivity of the cells to these pheromones. Only under unfavourable conditions (for example, poor growth media, small initial inocula and/or starved cells) is this self-promoting behaviour "visible".

For example, a dramatic reduction in the length of the lag phase of cultures of *Nitrosomonas europea* is mediated by N-(3-oxo-hexanoyl) homoserine lactone, and chorionic gonadotropin-like ligand (a 48kD protein) had similar growth-stimulating activity for *Xanthomonas maltophila*. A number of mammalian hormones (including peptide and steroid hormones as well as cytokines) have also been shown to exhibit

potent growth-stimulating activities for both Gram-positive and Gram-negative bacteria.

Latency and resuscitation

The ability of a microbial cell to grow and divide on a nutrient agar plate constitutes the benchmark method for determining the number of living cells in a sample of interest. However, it is also widely recognised that, especially in nature, the distinction between life and non-life is not absolute; many cells may exist in "dormant" or "moribund" forms or states and will not produce colonies on nutrient media (i.e. are "non-culturable"). However, these dormant or latent cells are not dead: they can be returned, by a process known as resuscitation, to a state of viability/ culturability.

For example, it is known that cells of the (high-G+C Gram-positive) bacterium *Micrococcus luteus* can enter a state of true dormancy from which they may be resuscitated by culture supernatants, even in the absence of any 'initially viable' cells.

The latent state has profound medical implications: many pathogenic bacteria (including pathogenic mycobacteria such as *M. tuberculosis*) are known to persist for extended periods in latent states in a host organism. Indeed, tuberculosis is a re-emergent infection of great concern, and it is recognised in particular that the causative organism (*Mycobacterium tuberculosis*) can lie dormant (remain latent) in patients and carriers for periods of years.

The latent state also has important commercial implications, since it complicates many laboratory methods for the detection, cultivation and enumeration of bacteria (for example in the food and healthcare industries).

There is therefore a pressing need to understand the physiological bases of latency and resuscitation.

Summary of the invention

The present invention is based, at least in part, on the discovery of a new class of pheromones which stimulate the resuscitation of bacteria after true dormancy. This "resuscitation factor" (herein embraced by the term "RP-factor") may exhibit activity at picomolar concentrations (implying a non-nutritional role). The elucidation of the structure of the pheromones at the amino acid sequence level has also permitted the present inventors to describe a larger family of proteins, some members of which act more broadly as regulators of cellular growth or replication and not necessarily as resuscitation promoting factors. Further sequence comparisons have also led to the identification of the cognate receptors, at least some of which share certain sequence similarities with their cognate RP-factors.

Thus, in a first aspect of the present invention there is provided an isolated RP-factor.

RP-factors

The term "RP-factor" is used herein to encompass any representative of that family of substances the members of which are capable of resuscitating dormant, moribund or latent cells (e.g. dormant bacterial cells). In addition, the RP-factors of the invention may also exhibit growth-stimulatory activity with respect to growing cells (e.g. growing bacterial cells), and/or may be competent to reduce the lag time of cell (e.g. bacterial cell) cultures. The resuscitation activity (and optionally also the growth-stimulatory activity or lag-time reducing activity) of the RP-factor may be specific for a particular (bacterial) cell (e.g. specific for one or more pathogenic mycobacterial, or may be non-specific. Specificity may be manipulated for example by engineering (e.g. by mutagenesis or chimaerisation, as herein described) of the specificity-determining domain(s) of the RP-factor or by replacement of the signalling domain.

The term "RP-factor" is also used herein in a somewhat broader sense to encompass polypeptides which are expressed by bacteria and which regulate (e.g. promote, trigger, prevent or impair) the growth or multiplication of a cell (the "target cell") by acting as signalling moieties in conjunction with (e.g. by binding to) cognate cellular receptors. Such polypeptides may be referred to herein as bacterial cytokines.

The RP-factors of the invention therefore inlcude bacterial cytokines which may or may not be capable of resuscitating dormant, moribund or latent cells (e.g. dormant bacterial cells) and/or exhibit growth-stimulatory activity with respect to growing cells (e.g. growing bacterial cells). They may or may not also be competent to reduce the lag time associated with the growth of cell (e.g. bacterial cell) cultures. Moreover, some bacterial cytokines which fall within the scope of the term "RP-factor" as defined herein may even prevent or impair the growth of the target cells (particularly where the target cells are eukaryotic (e.g. mammalian) cells).

The RP-factors of the invention may fall into at least two functional classes: autosignalling factors and allosignalling factors. Autosignalling factors act to regulate the growth of the bacterial cell in which they were expressed (i.e. they act as bacterial autocrine factors), while allosignallers act to regulate the growth of other cells (i.e. they act as bacterial paracrine factors). Autosignalling factors therefore act as self-regulators of bacterial cell growth, and may be essential for viability and/or growth. Some RP-factors may function as both auto- and allosignalling cytokines.

Allosignalling factors may exhibit a range of different specificities. Some may act solely on other bacterial cells of the same species as the cell in which they were expressed ("homoactive" factors), while others may act on cells of one or more other species ("heteroactive" factors). Heteroactive factors may exhibit a broad range of specificity: they may act on several different species (for example, in a genus-specific manner), or may be species-specific. Some heteroactive bacterial factors may act on eukaryotic cells, and may be specific for particular cell-types. For example, some heteroactive bacterial cytokines (particularly those produced by certain pathogens) may act on

mammalian cells (e.g. mammalian epithelial, endothelial or immune cells), and may be tissue- or cell-type specific.

Notwithstanding the above explanation, it is postulated that the specificity of at least some RP-factors may be concentration dependent. In these cases, the specificity of any given RP-factor falls within a continuum, so that an autosignalling RP-factor may mediate cross-talk and so exhibit allosignalling activity when present at sufficiently high concentrations. Similarly, allosignalling RP-factors may exhibit homo- or heteroactivity depending on concentration.

The RP-factor may be translocated through the cell membrane, whereafter it may be secreted into the surrounding environment or remain associated with the surface of the cell. Thus, at least two classes of RP-factor may exist: secreted and non-secreted. The secreted RP-factors are characterised by the presence of a secretory signal sequence (the presence of which is readily recognised by those skilled in the art on the basis of the presence of DNA and/or amino acid sequence motifs). The non-secreted RP-factors may be cell-associated or cytosolic factors. Both classes of RP-factor may exist in a single cellular source (e.g. in a single bacterial source). Both classes of RP-factor find application in the invention.

Non-secreted RP-factors may act in at least four different ways: (a) as a membrane-anchored juxtacrine factor mediating a growth regulating signal between two different cells in close physical proximity or contact; and/or (b) as an intercellular signalling moiety upon cleavage by an enzyme (e.g. a convertase, as herein defined) which releases a soluble signalling moiety into the extracellular milieu; and/or (c) as an autocrine factor *via* binding to cognate receptors located on the surface of the cell in which the non-secreted factor is expressed or acting entirely intracellularly; and/or (d) as a cognate receptor for another non-secreted or secreted RP-factor.

Thus, the RP-factors of the invention may include the nine factors identified by the sequences shown in Fig. 1A and the five factors identified by those shown in Fig 1B, together with their species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms (*vide infra*).

Preferably, the RP-factors of the invention are species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms of any one of the nine factors identified by the sequences shown in Fig. 1A and the five factors identified by those shown in Fig. 1B.

The RP-factors may be synthesised in the form of a precursor which is processed to produce a mature form. Such processing may proceed *via* various intermediate (pro-) forms. Such precursors, intermediate forms and mature proteins are all intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise. As used herein, the term "pro-RP-factor" specifically defines any of various precursors (which may or may not be active) of a mature RP-factor.

The processing may comprise proteolytic cleavage and/or secretion. The precursors may be inactive, and become active on processing as a mature form. The precursors may comprise proteins having secretory leader sequences which are removed during secretion (pre- forms). Such forms are herein referred to as "pre-RP-factor or pre-pro-RP-factors". As explained above, such pre- or prepro- forms are also intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise.

Processing may be attendant on the binding of an RP-factor precursor to a cognate receptor. Such receptors may then directly (or indirectly) cleave the precursor to produce a more mature form of the RP-factor. Such processing may occur as a cascade, involving several receptor-processing complexes, and so ultimately result in the production of a mature RP-factor which then acts as a signalling moiety by binding to a terminal (signal transducing) receptor.

In such processing, the proximal (or intermediate) receptors may function as convertases, and the terminal receptor as a signal transducer. However, a receptor may function as both a convertase and a signal transducer. As used herein, the term "convertase" is intended to define a molecule which binds an RP-factor precursor and (directly or indirectly) processes it to produce a more mature form. They may, for example, have protease activity.

The receptors/convertases discussed above may be disposed at the cell surface (e.g. membrane bound), cytosolic or extracellular.

Preferably, the RP-factor is derived from a bacterium (e.g. a pathogenic bacterium). Particularly preferred are RP-factors derived from high G+C Gram-positive bacteria. However, the inventors have also discovered RP-factor family members in representatives of the low G+C Gram-positive organisms, including *Bacillus subtilis* and clostridia. Thus, RP-factors derived from low G+C Gram-positive bacteria (e.g. pathogenic low G+C Gram-positive bacteria) are also preferred according to the invention. Examples of the latter include: *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp. and *Lactobacillus* spp..

The invention also contemplates homologues, allelic forms, species variants, derivatives, muteins or equivalents of the RP-factors and RP-factor receptors/convertases of the invention.

Preferably, the homologues, derivatives, muteins or equivalents of the RP-factor of the invention have at least 20% identity with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% identity, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

The homologues, derivatives, muteins or equivalents of the RP-factor of the invention may have at least 25% homology with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% homology with any one of the particular amino acid sequences shown in Fig. 1A and 1B.

The invention also contemplates chimaeric RP-factors. These are factors which comprise one or more heterologous domains. In this context, a heterologous domain is a portion of an RP-factor which is derived from a different RP-factor to that from which the other domain(s) with which it is associated are derived. Such chimaeric RP-factors find particular utility in applications where the specificity and/or activity of the RP-factor is manipulated or altered.

The invention also contemplates all individual functional domains of the RP-factors of the invention as separate and independent entities.

The invention also contemplates recombinant RP-factor. As used herein, the term "recombinant" is intended to define material which has been produced by that body of techniques collectively known as "recombinant DNA technology" (for example, using the nucleic acid, vectors and or host cells described *infra*).

Cognate receptors

In some cases, the cognate cellular receptor is a cell surface receptor: in other cases, it is a cytosolic receptor with which the cytokine interacts after uptake by the target cell. The receptors with which the RP-factors and/or bacterial cytokines of the invention interact may share certain structural motifs with the RP-factors/cytokines themselves. In particular, the receptors may contain a ligand binding domain which is structurally similar to the signalling domain of the cognate RP-factor/cytokine.

The receptors may also comprise a membrane anchor domain and a wall spanning domain.

Preferably, the cognate receptor comprises a receptor domain as hereinbelow defined and/or a wall spanning domain domain as hereinbelow defined and/or a membrane anchor.

Particularly preferred are cognate receptors comprising the amino acid sequence of MtubZ94752 as shown in Fig. 1A or the amino acid sequence of YabE from *B. subtilis* as shown in Fig. 1B.

The cognate receptors may also comprise derivative or equivalent sequences of amino

acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The cognate receptors may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with the amino acid sequence of MtubZ94752 as shown in Fig. 1A or the amino acid sequence of YabE from *B. subtilis* as shown in Fig. 1B, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

RP-factor/cognate receptor domain structure

The RP-factors of the invention (including the bacterial cytokines as also defined herein) and their cognate receptors may comprise a plurality of discrete domains. These domains may be functionally and/or structurally distinct.

The RP-factors of the invention may be characterised by the presence of at least two functional domains: a secretory signal sequence (which may be wholly or partially absent in the active form of the factor) and a signalling domain. The signalling domain may fall into one of at least two distinct classes described in more detail *infra*.

Many RP-factors also comprise a third functional domain which mediates a physical association with the surface of the target cell (hereinafter referred to as the "localizing domain" and described in more detail *infra*).

The RP-factors of the invention may further comprise a specificity-determining domain, which may function in conjunction with the signalling domain.

Non-secreted RP-factors may further comprise a wall-spanning domain (described in more detail *infra*) and/or a membrane anchor.

The gross structure and/or amino acid sequence of the aforementioned domains may vary considerably. In particular, the structure of the surface localizing domain may differ according to the structure of the cell-wall of the target cell. For example, the surface localizing domain may fall into one of at least two distinct classes: class I (which may act on peptidoglycan) and class II (which may act on the outer lipid envelope found in mycobacteria).

The cognate receptors of the invention may be characterised by the presence of at least two functional domains: a receptor domain and a wall spanning domain. They may also comprise a membrane anchor. The receptor domain may be structurally similar to the signalling domain of the cognate RP-factor (as described in more detail *infra*).

Receptor/signalling domain, class I

This domain may be associated with RP-factors from high G+C Gram-positive bacteria (such as mycobacteria and *Micrococcus* spp.) and/or their cognate receptors. When present on RP-factors, the domain may be involved in receptor binding, and may for example bind a structurally similar domain on a cognate receptor. Thus, when present as part of an RP-factor of the invention, the domain is termed the "signalling domain", and when present in the cognate receptor, the domain is termed the "receptor domain".

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks in any one of the 9 sequences set out in Figure 1A.

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks and dots in any one of the 9 sequences set out in Figure 1A.

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 9 sequences set out in Figure 1A.

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case type in any one of the 9 sequences set out in Figure 1A.

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Receptor/signalling domain, class II

This domain may be associated with RP-factors from low G+C Gram-positive bacteria (such as bacilli and clostridia) and/or their cognate receptors. When present on RP-factors, the domain may be involved in receptor binding, and may for example bind a structurally similar domain on a cognate receptor. Thus, when present as part of an RP-factor of the invention, the domain is termed the "signalling domain", and when present in the cognate receptor, the domain is termed the "receptor domain".

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks in any one of the 5 sequences set out in Figure 1B(B).

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks and dots in any one of the 5 sequences set out in Figure 1B(B).

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 5 sequences set out in Figure 1B(B).

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case type in any one of the 5 sequences set out in Figure 1B(B).

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Wall spanning domain

This domain may be associated with non-secreted RP-factors (e.g. cell-associated RP-factors or RP-factors which act as juxtacrine factors) and with the cognate receptors of the RP-factors of the invention. When present, the domain is involved in mediating an interaction with the cell wall such that the RP-factor/receptor as a whole may span it. The wall spanning domain may therefore be bounded by cytosolic and extracellular regions *in vivo*. The domain is often associated with a membrane anchor, the two structural elements acting in concert to maintain the RP-factor/receptor at the cell surface.

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case and indexed by hashes (#) in any one of the 5 sequences set out in Figure 1B(A).

In preferred embodiments, the domain may comprise a sequence of amino acid residues,

the identity and relative positions of which correspond to those residues presented in upper case and indexed by hashes and dots in any one of the 5 sequences set out in Figure 1B(A).

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 5 sequences set out in Figure 1B(A).

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues presented in upper case type in any one of the 5 sequences set out in Figure 1B(A).

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Localizing domain, class I

This domain may be present in secreted RP-factors, and may mediate a physical association with the surface of the target cell by acting to bind peptidoglycan or some other surface component(s). It may therefore act to increase the local concentration of the cytokine at the target cell surface, so promoting activity by increasing the local concentration of RP-factor in the immediate vicinity of the cognate receptor. Localizing domains may therefore be a characteristic feature of allosignalling bacterial cytokines, and may be absent in autosignalling factors or *vice versa*. For example, when present in autosignalling factors, localizing domains may act to retain the factor at or near the cell surface after secretion through the cell membrane.

When present, the localizing domain may confer important binding properties on the RP-factor, whereby binding to cognate receptor is biphasic and characterised by a primary (relatively unspecific) association with the cell surface followed by a secondary (relatively highly specific) association with the cognate receptor.

The domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks in any one of the 10 sequences set out in Figure 1C.

In preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues indexed by asterisks and dots in any one of the 10 sequences set out in Figure 1C.

In particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues highlighted in bold upper case type in any one of the 10 sequences set out in Figure 1C.

In more particularly preferred embodiments, the domain may comprise a sequence of amino acid residues, the identity and relative positions of which correspond to those residues set out in any one of the 10 sequences set out in Figure 1C.

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

Localizing domain, class II

This domain may be present in secreted RP-factors, and may mediate a physical association with the surface of the target cell by acting to bind the outer lipid envelope present in mycobacteria. It may therefore act to increase the local concentration of the cytokine at the target cell surface, so promoting activity by increasing the local concentration of RP-factor in the immediate vicinity of the cognate receptor. Localizing domains may therefore be a characteristic feature of allosignalling bacterial cytokines, and may be absent in autosignalling factors.

When present, the localizing domain may confer important binding properties on the RP-factor, whereby binding to cognate receptor is biphasic and characterised by a primary (relatively unspecific) association with the cell surface followed by a secondary (relatively highly specific) association with the cognate receptor.

The domain may comprise an alanine plus proline-rich segment, such as one or more of the amino acid motifs 'A', A, B, B', C, 'C, D, D* and D' (any one of which may be tandemly repeated) as set out in Figure 1D.

In preferred embodiments, the domain may comprise a sequence of amino acid residues corresponding to residues 158-322 of MtubMTV043 as shown in Figure 1D or to that of residues 45-112 of MtubMTV008 as shown in Figure 1A.

The domain may also comprise derivative or equivalent sequences of amino acid residues which are as defined above but in which amino acids have been added, deleted or substituted (e.g. conservatively substituted), provided that biological activity (e.g. signalling or ligand-binding activity) is substantially retained.

The domain may also comprise derivative or equivalent sequences of amino acid residues which have at least 20% identity or homology with any one of the particular amino acid sequences defined above, for example at least 30% identity or homology, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity or homology therewith.

The term "isolated" is used herein to indicate that the factor exists in a physical milieu distinct from that in which it occurs in nature. For example, the isolated factor may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs. The absolute level of purity is not critical, and those skilled in the art can readily determine appropriate levels of purity according to the use to which the factor is to be put.

In many circumstances, the isolated factor will form part of a composition (for example a more or less crude extract containing many other proteins and substances), buffer system or pharmaceutical excipient, which may for example contain other components (including other proteins, such as albumin).

In other circumstances, the isolated protein may be purified to essential homogeneity, for example as determined by PAGE or column chromatography (for example HPLC or mass spectrometry). In preferred embodiments, the isolated RP-factor of the invention is essentially the sole active RP-factor in a given composition. Particularly preferred are compositions in which an RP-factor (or a particular species, homologue, mutein, derivative or equivalent thereof) is present as the sole active ingredient in a pharmaceutical composition.

The RP-factor for use in the invention need not be isolated in the sense defined above, however. For example, more or less crude culture supernatants (e.g. "spent" medium) may contain sufficient concentrations of RP-factor for use in several applications. Preferably, such supernatants are fractionated and/or extracted (see below), but in many circumstances they may be used without pretreatment. They are preferably derived from spent media used to culture RP-factor-producing microorganisms (for example, the bacterial sources described *infra*). The supernatants are preferably sterile. They may be treated in various ways, for example by concentration, filtration, centrifugation, spray drying, dialysis and/or lyophilisation. Conveniently, the culture supernatants are simply centrifuged to remove cells/cell debris and filtered.

Such supernatants find utility in diagnostic kits and methods, for example in the diagnostic kits and methods described *infra*. They also find utility in the recovery from various samples of culturable microorganisms (e.g. from soil, food, marine, freshwater,

or tissue samples) or from samples taken from an organism (e.g. a human or animal).

The culture supernatants may also be used as supplements in various culturing substrates, for example in culture or transport media. The culture medium may take any convenient form, such as for example agar plates, broths, slopes, coated dipsticks, coated probes, membranes, coated or filled wells or films. The medium may be a defined or complex medium, and may contain indicator dyes to facilitate identification of cultured microorganisms. Preferably, the medium is suitable for the culturing or transport of bacteria, for example *Mycobacterium* spp..

The term "isolated" as applied to the other materials of the invention (for example, the genes and other nucleic acids encoding the RP-factor and their cognate receptors/convertases) is to be interpreted mutatis mutandis. Thus, as applied to nucleic acid (e.g. RNA or DNA or (structural) genes), the isolated nucleic acid may be present in any of a wide variety of vectors and in any of a wide variety of host cells (or other milieu, such as buffers, viruses or cellular extracts).

The term "family", as applied to the proteins of the invention, is used herein to indicate a group of proteins which share substantial sequence similarities, either at the level of the primary sequence of the proteins themselves, or at the level of the DNA encoding them. The sequence similarities may extend over the entire protein/gene, or may be limited to particular regions or domains. Similarities may be based on nucleotide/amino acid sequence identity as well as similarity (for example, those skilled in the art recognise certain amino acids as similar, and identify differences based on switches of similar amino acids as conservative changes). Some members of a protein family may be related in the sense that they share a common evolutionary ancestry, and such related proteins may herein be referred to as homologues. The members of a protein family do not necessarily share the same biochemical properties or biological functions, though their similarities are usually reflected in common functional features (such as effector binding sites and substrates).

The criteria by which protein families are recognised are well-known in the art, and include computer analysis of large collections of sequences at the level of DNA and protein as well as biochemical techniques such as hybridisation analysis and enzymatic assays (see for example Pearson and Lipman (1988), PNAS USA, 85: 2444).

Thus, the RP-factors of the invention include the factors shown in Fig. 1A and 1B, together with their species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms (*vide infra*). Preferably, the RP-factors of the invention are species variants, allelic forms, homologues, derivatives, muteins and corresponding secreted/nonsecreted forms of any one of the proteins represented in Fig. 1A and Fig. 1B.

The RP-factors may be synthesised in the form of a precursor which is processed to produce a mature form. Such processing may proceed *via* various intermediate (pro-)

forms. Such precursors, intermediate forms and mature proteins are all intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise. As used herein, the term "pro-RP-factor" specifically defines any of various precursors (which may or may not be active) of a mature RP-factor.

The processing may comprise proteolytic cleavage and/or secretion. The precursors may be inactive, and become active on processing as a mature form. The precursors may comprise proteins having secretory leader sequences which are removed during secretion (pre- forms). Such forms are herein referred to as "pre-RP-factor or pre-pro-RP-factors". As explained above, such pre- or prepro- forms are also intended to be covered by the term "RP-factor" as used herein, except where indicated otherwise.

Processing may be attendant on the binding of an RP-factor precursor to a cognate receptor. Such receptors may then directly (or indirectly) cleave the precursor to produce a more mature form of the RP-factor. Such processing may occur as a cascade, involving several receptor-processing complexes, and so ultimately result in the production of a mature RP-factor which then acts as a signalling moiety by binding to a terminal (signal transducing) receptor.

In such processing, the proximal (or intermediate) receptors may function as convertases, and the terminal receptor as a signal transducer. However, a receptor may function as both a convertase and a signal transducer. As used herein, the term "convertase" is intended to define a molecule which binds an RP-factor precursor and (directly or indirectly) processes it to produce a more mature form. They may, for example, have protease activity.

The receptors/convertases discussed above may be disposed at the cell surface (e.g. membrane bound), cytosolic or extracellular.

Preferably, the RP-factor is derived from a bacterium (e.g. a pathogenic bacterium). Particularly preferred are RP-factors derived from high G+C Gram-positive bacteria.

The term "derived from" as applied to a defined source is intended to define not only a source in the sense of it being the *physical* origin for the material, but also to define material which has structural and/or functional characteristics which correspond to those of material which does originate from the reference source. Thus, a protein "derived from" a given source need not necessarily have been purified from that source.

The term "high G+C Gram-positive bacterium" is a term of art defining a particular class of evolutionarily related bacteria. The class includes *Micrococcus* spp. (e.g. *M. luteus*), *Mycobacterium* spp. (for example a fast- or slow-growing mycobacterium, e.g. *M. tuberculosis*, *M. leprae*, *M. smegmatis* or *M. bovis*), *Streptomyces* spp. (e.g. *S. rimosus* and *S. coelicolor*) and *Corynebacterium* spp. (e.g. *C. glutamicum*). Preferred according to the invention are RP-factors/cognate receptors/convertases derived from mycobacteria ("mycobacterial RP-factors/RP-factor receptors/convertases").

The invention also contemplates homologues, allelic forms, species variants, derivatives, muteins or equivalents of the RP-factors and RP-factor receptors/convertases of the invention.

The term "homologue" is used herein in two distinct senses. It is used *sensu stricto* to define the corresponding protein from a different organism (i.e. a species variant), in which case there is a direct evolutionary relationship between the protein and its homologue. This may be reflected in a structural and functional equivalence, the protein and its homologue performing the same role in each organism.

The term is also used herein *sensu lato* to define a protein which is structurally *similar* (i.e. not necessarily related and/or structurally and functionally equivalent) to a given (reference) RP-factor. In this sense, homology is recognised on the basis of purely structural criteria by the presence of amino acid sequence identities and/or conservative amino acid changes (as set out by Dayhoff *et alia, Atlas of protein structure* vol. 5, National BioMed Fd'n, Washington D.C., 1979).

For the purposes of the invention, homologues may be recognised as those proteins the corresponding DNAs of which are capable of specifically or selectively cross-hybridising, or which can cross-hybridise under selective, appropriate and/or appropriately stringent hybridisation conditions.

The term "selectively or specifically (cross)hybridisable" in this context indicates that the sequences of the corresponding ssDNAs are such that binding to a unique (or small class) of homologous sequences can be obtained under more or less stringent hybridisation conditions. This method of the invention is not dependent on any particular hybridisation conditions, which can readily be determined by the skilled worker (e.g. by routine trial and error or on the basis of thermodynamic considerations).

Preferably, the homologues, derivatives, muteins or equivalents of the RP-factor of the invention have at least 20% identity with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% identity, for example at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

The homologues, derivatives, muteins or equivalents of the RP-factor of the invention may have at least 25% homology with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

Particularly preferred are homologues, derivatives, muteins or equivalents of the RP-factor of the invention which have at least 30% homology, for example at least 35%,

40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% homology with any one of the particular amino acid sequences shown in Fig. 1A or Fig. 1B.

The term "derivative" as applied herein to the proteins (e.g. the RP-factors or RP-factor receptors/convertases) of the invention is used to define proteins which are modified versions of the proteins of the invention. Such derivatives may include fusion proteins, in which the proteins of the invention have been fused to one or more different proteins or peptides (for example an antibody or a protein domain conferring a biochemical activity, to act as a label, or to facilitate purification).

The derivatives may also be products of synthetic processes which use a protein of the invention as a starting material or reactant.

The term "mutein" is used herein to define proteins that are mutant forms of the proteins of the invention, i.e. proteins in which one or more amino acids have been added, deleted or substituted. The muteins of the invention therefore include fragments, truncates and fusion proteins (e.g. comprising fused immunoglobulin, receptor, convertase or enzyme moieties).

The muteins of the invention also include proteins in which mutations have been introduced which effectively promote or impair one or more activities of the protein, for example mutations which promote or impair the function of a receptor, a recognition sequence or an effector binding site.

Muteins may be produced by any convenient method. Conveniently, site-directed mutagenesis with mutagenic oligonucleotides may be employed using a double stranded template (pBluescript KS II construct containing the RP-factor or RP-factor receptor/convertase gene), (e.g. Chameleon™ or QuikChange™ - Stratagene™). After verifying each mutant derivative by sequencing, the mutated gene is excised and inserted into a suitable vector so that the modified protein can be over-expressed and purified.

Preferred mutant forms are truncates consisting (or consisting essentially) of the RP-factor signalling domain or the RP-factor specificity-determining factor, or of the ligand binding domain of the RP-factor receptor, or combinations of two or more of the foregoing.

The invention also contemplates chimaeric RP-factors. These are factors which comprise one or more heterologous domains. In this context, a heterologous domain is a portion of an RP-factor which is derived from a different RP-factor to that from which the other domain(s) with which it is associated are derived. Such chimaeric RP-factors find particular utility in applications where the specificity and/or activity of the RP-factor is manipulated or altered.

Useful in the construction of such chimaeric RP-factors are DNA fragments or cassettes consisting essentially of DNA encoding selected domains (for example, the signalling domain or the specificity-determining domain), the fragment or cassette optionally being bounded by one or more restriction endonuclease cleavage sites or cloning sites. The invention also contemplates concatenated domain cassettes, as well as mutant RP-factor structural genes which have cloning sites (e.g. one or more restriction endonuclease cleavage sites) located in one or more interdomain regions.

The term equivalent as used herein and applied to the materials of the invention defines materials (e.g. proteins, DNA etc.) which exhibit substantially the same functions as those of the materials of the invention while differing in structure (e.g. nucleotide or amino acid sequence). Such equivalents may be generated for example by identifying sequences of functional importance (e.g. by identifying conserved or canonical sequences or by mutagenesis followed by functional assay), selecting an amino acid sequence on that basis and then synthesising a peptide based on the selected amino acid sequence. Such synthesis can be achieved by any of many different methods known in the art, including solid phase peptide synthesis (to generate synthetic peptides) and the assembly (and subsequent cloning) of oligonucleotides.

The homologues, fragments, muteins, equivalents or derivatives of the proteins of the invention may also be defined *inter alia* as those proteins which cross-react with antibodies to the proteins of the invention, and in particular which cross-react with antibodies directed against any of the specific proteins shown Fig. 1A or Fig. 1B.

The invention also contemplates all individual functional domains of the RP-factors of the invention as separate and independent entities.

The invention also contemplates recombinant RP-factor. As used herein, the term "recombinant" is intended to define material which has been produced by that body of techniques collectively known as "recombinant DNA technology" (for example, using the nucleic acid, vectors and or host cells described *infra*).

The invention also contemplates a pharmaceutical composition (e.g. a vaccine) comprising the RP-factor or RP-factor receptor/convertase (or homologue, species variant, allelic form, derivative, mutein or equivalent thereof) of the invention.

A pharmaceutical composition is a solid or liquid composition in a form, concentration and level of purity suitable for administration to a patient (e.g. a human or animal patient) upon which administration it can elicit the desired physiological changes. The vaccines of the invention may include any suitable adjuvant (e.g. Freund's adjuvant, BCG or BCG extracts).

In another aspect, the invention relates to a pharmaceutical composition comprising the material of the invention which is: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form

suitable for local or systemic administration.

In another aspect, the invention relates to an antibody (or antibody derivative) specific for the RP-factor (or homologue, derivative, mutein or equivalent thereof) of the invention.

The antibody is preferably in a form suitable for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or formulated in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration. The antibody may be labelled and/or immortalised and/or conjugated to another moiety, and such embodiments find particular utility in diagnostic applications.

According to another aspect of the invention there is provided an isolated or recombinant RP-factor receptor.

The receptor/convertase may be derived from any of the sources hereinbefore described, for example from a bacterial source (e.g. a pathogenic bacterial source). Such sources include high G+C Gram-positives, *Micrococcus* spp. (e.g. *M. luteus*); or *Mycobacterium* spp. (for example a fast- or slow-growing mycobacterium, e.g. *M. tuberculosis*, *M. leprae*, *M. smegmatis* or *M. bovis*); or *Streptomyces* spp. (e.g. *S. rimosus* and *S. coelicolor*); or *Corynebacterium* spp. (e.g. *C. glutamicum*).

The invention also contemplates homologues, derivatives, muteins or equivalents of the receptors/convertases of the invention, as well as recombinant RP-factor receptors/convertases (as hereinbefore defined).

The invention also contemplates a pharmaceutical composition (e.g. a vaccine) comprising the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) of the invention.

Preferably, the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) or pharmaceutical composition is: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated is an antibody (or antibody derivative) specific for the receptor/convertase (or homologue, derivative, mutein or equivalent thereof) of the invention. The antibody may be: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated is an RP-factor antagonist or inhibitor.

Preferably, the antagonist or inhibitor comprises: (a) the antibody of the invention; and/or (b) the receptor/convertase of the invention; and/or (c) an RP-factor mutein

comprising an RP-factor specificity-determining domain, which for example lacks a functional signalling domain. The receptor may function as an antagonist or inhibitor if administered in soluble form, where it may act as a sink for soluble RP-factor. Preferably, modified receptors consisting of the receptor domain (and lacking the membrane anchor and wall spanning domain) are used as inhibitors or antagonists. Such derivatives may exhibit higher solubility.

The antagonist or inhibitor of the invention is preferably: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

Also contemplated by the invention is an RP-factor agonist, activator or mimetic. Preferably, the agonist, activator or mimetic comprises: (a) the RP-factor receptor/convertase antibody as herein described; and/or (b) an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; and/or (c) an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or (d) operably coupled combinations of any of (a)-(c).

The agonist, activator or mimetic may be: (a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or (b) formulated in a pharmaceutical excipient, a unit dosage form, in a form suitable for local or systemic administration or in admixture with an antibiotic.

Preferably, the agonist, activator or mimetic may be for use in adjunctive therapy (for example formulated or presented in combination with an antimicrobial agent, e.g. an antibiotic).

The invention also contemplates isolated nucleic acid encoding the RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) or RP-factor receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) of the invention. The nucleic acids of the invention therefore embrace DNA having any sequence so long as it encodes the proteins of the invention. It will be appreciated by those skilled in the art that as a result of degeneracy in the genetic code, any particular amino acid sequence of the invention may be encoded by many different sequences. Thus, the nucleic acid sequence may be selected or optimised, e.g. with respect to the codon usage in any particular host cell.

The invention also contemplates vectors (e.g. an expression vector) comprising the nucleic acid of the invention. The nature of the vector is not critical to the invention. Any suitable vector may be used, including plasmid, virus, bacteriophage, transposon, minichromosome, liposome or mechanical carrier.

The expression vectors of the invention are DNA constructs suitable for expressing DNA which encodes the desired protein product (e.g. RP-factor or RP-factor receptor) which may include: (a) a regulatory element (e.g. a promoter, operator, activator, repressor

and/or enhancer), (b) a structural or coding sequence which is transcribed into mRNA and (c) appropriate transcription, translation, initiation and termination sequences. They may also contain sequence encoding any of various tags (e.g. to facilitate subsequent purification of the expressed protein, such as affinity (e.g. His) tags).

Particularly preferred are vectors which comprise an expression element or elements operably linked to the DNA of the invention to provide for expression thereof at suitable levels. Any of a wide variety of expression elements may be used, and the expression element or elements may for example be selected from promoters, enhancers, ribosome binding sites, operators and activating sequences. Such expression elements may comprise an enhancer, and for example may be regulatable, for example being inducible (*via* the addition of an inducer).

As used herein, the term "operably linked" refers to a condition in which portions of a linear DNA sequence are capable of influencing the activity of other portions of the same linear DNA sequence. For example, DNA for a signal peptide (secretory leader) is operably linked to DNA for a polypeptide if it is expressed as a precursor which participates in the secretion of the polypeptide; a promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The vector may further comprise a positive selectable marker and/or a negative selectable marker. The use of a positive selectable marker facilitates the selection and/or identification of cells containing the vector.

Also contemplated by the invention are host cells comprising the vector of the invention. Any suitable host cell may be used, including prokaryotic host cells (such as *Escherichia coli*, *Streptomyces* spp. and *Bacillus subtilis*) and eukaryotic host cells.

In another aspect, the invention provides a culture or transport medium comprising the RP-factor (or homologue, derivative, mutein or equivalent thereof) of the invention. The culture medium may take any convenient form, such as for example agar plates, broths, slopes, coated dipsticks, coated probes, membranes, coated or filled wells or films. The medium may be a defined or complex medium, and may contain indicator dyes to facilitate identification of cultured microorganisms. Preferably, the medium is suitable for the culturing or transport of bacteria, for example *Mycobacterium* spp. *Streptomyces* spp. and *Corynebacterium* spp.

The invention also contemplates a nucleic acid probe comprising nucleic acid complementary to the nucleic acids of the invention. Such probes are preferably selectively hybridisable with nucleic acid encoding the proteins (e.g. the RP-factors of RP-factor receptors/convertases) of the invention. They are conveniently single stranded DNA or RNA probes.

The invention also contemplates a diagnostic kit comprising the factor (or homologue,

derivative, mutein or equivalent thereof), receptor, antibody, probe or culture medium of the invention.

In another aspect, the invention contemplates antisense DNA corresponding to the nucleic acid encoding the RP-factor or RP-factor receptor/convertase of the invention.

The invention also contemplates a process for producing an antimicrobial drug comprising the steps of: (a) providing an RP-factor receptor; (b) providing candidate drugs; (c) screening the candidate drugs by contacting the RP-factor receptor/convertase with one of the candidate drugs and determining the affinity of the candidate drug for the RP-factor receptor, wherein the affinity is an index of antimicrobial activity, and optionally (d) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (c).

Preferably, the process for producing an antimicrobial drug comprises the steps of: (a) providing an RP-factor receptor/convertase; (b) providing a candidate drug; (c) providing an RP-factor; (d) screening the candidate drugs by contacting the RP-factor receptor/convertase with one of the candidate drugs in the presence of the RP-factor, and then determining the ability of the candidate drug to compete non-productively with the RP-factor for binding to the RP-factor receptor, wherein the competitive binding ability is an index of antimicrobial activity, and optionally (e) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (d).

The invention also covers an antimicrobial drug produced by (or obtainable by) the processes of the invention, and also derivatives thereof.

Also contemplated by the invention is a method for determining the microbiological quality of a product (e.g. a foodstuff, pharmaceutical preparation or medical product) comprising the step of contacting a sample of the product with an RP-factor (for example, an RP-factor as hereinbefore defined). In such methods, the RP-factor preferably forms part of a nutrient composition (e.g. a plate, broth, film or dipstick).

In another aspect, the invention relates to a method of culturing bacterial (e.g. mycobacterial) cells, comprising the step of incubating the cells in a culture medium containing an RP-factor (for example, an RP-factor as hereinbefore defined).

Also contemplated by the invention is an *ex vivo* method of diagnosis, comprising the step of contacting a biological sample with an RP-factor (for example, an RP-factor as hereinbefore defined).

The diagnostic method of the invention preferably includes the step of incubating the culture or transport medium of the invention to permit growth of cells in the biological sample (e.g. bacterial cells).

Also contemplated by the invention is a method of: (a) stimulating the growth of a microorganism; and/or (b) resuscitating a dormant, moribund or latent microorganism; comprising the step of contacting the microorganism with an RP-factor (for example, an RP-factor as hereinbefore defined).

The invention also contemplates a process for producing the recombinant RP-factor or RP-factor receptor/convertase of the invention comprising the steps of: (a) culturing the host cell of the invention, and (b) purifying the factor or receptor/convertase from the cultured host cells (e.g. from a culture supernatant or cell fraction).

Also contemplated by the invention is a process for producing the recombinant RP-factor or receptor/convertase of the invention comprising the steps of: (a) probing a gene library with a nucleic acid probe which is selectively hybridisable with the cognate structural gene to produce a signal which identifies a gene that selectively hybridises to the probe; (b) expressing the gene identified in step (a) (for example by cloning into a host cell, e.g. according to the process as hereinbefore defined) to produce the factor or receptor.

Also covered is a recombinant RP-factor or receptor/convertase obtainable by the above-described process.

Medical applications

The invention permits the isolation, synthesis and rational design of a wide range of novel medicaments and pharmaceuticals for use in therapy, prophylaxis and diagnosis.

The various forms of therapy, prophylaxis and diagnosis in which the materials of the invention find application may involve changing, breaking or perturbing the resuscitation (RP-factor) signal transduction pathway of one or more infecting pathogens.

Thus, the materials of the invention find general application as antimicrobial agents, for example as antibacterial agents. They may therefore be used in the treatment, prophylaxis or diagnosis of microbial (e.g. bacterial) infections, particularly those infections associated with latency (e.g. mycobacterial infections).

Thus, the invention may for example be used to prevent, reduce or interfere with: (a) the resuscitation of a latent (or dormant) pathogen, and/or (b) the growth of a pathogen, and/or (c) the multiplication and spread of a pathogen; and/or (d) the activation of a latent infection (for example a latent bacterial (e.g. mycobacterial) infection).

In general, the materials of the invention may be used to treat conditions in which changing, breaking or perturbing the resuscitation (RP-factor) signal transduction pathway or blockading the RP-factor receptor/convertase associated with an infecting pathogen is indicated.

Particularly useful materials for use in such therapies/prophylactic methods include RP-factor antagonists or inhibitors. Such antagonists or inhibitors may comprise antibodies to the RP-factor or to the RP-factor receptor/convertase as herein defined; the RP-factor receptor/convertase of the invention; an RP-factor mutein, e.g. which comprises an altered RP-factor specificity-determining domain and/or which lacks a functional signalling domain.

RP-factor antibodies act to sequester and ultimately eliminate endogenous RP-factors in a patient bearing a latent microbial infection.

RP-factor receptor antibodies bind non-productively to the receptors associated with the infecting pathogen. Antibodies to the convertase inactivate (e.g. by steric inhibition) the convertase activity and so prevent maturation of the RP-factor. The antibodies may therefore competitively inhibit the binding of endogenous RP-factor to the receptors/convertases associated with the infecting pathogen. Alternatively, they may bind with high affinity (and/or essentially irreversibly) to the RP-factor receptors/convertases and so block RP-factor-ligand binding or RP-factor maturation. A similar activity is displayed by the RP-factor muteins having altered specificity and/or signalling activity.

In either case, the RP-factor-RP-receptor/convertase binding required for resuscitation of latent pathogens, growth of the pathogen and/or progression of the disease state is perturbed, reduced or abolished.

RP-factor receptors for use as therapeutics in such methods are uncoupled from the signal transduction pathway with which they are normally associated. Thus, they are preferably free (i.e. in soluble or dispersible) form and/or not membrane bound. In this way, effective circulating or systemic concentrations of the free RP-factor receptor can be established and maintained in a patient. In this form, the RP-factor receptors act as RP-factor sinks, and titrate out (and preferably ultimately eliminate) endogenous RP-factors in a patient bearing a latent microbial infection. The receptors therefore reduce or prevent activation of the (latent) pathogen and/or stimulation of pathogen growth, so slowing or halting the progression of the infection.

In another aspect, the invention may be used to resuscitate or assist in resuscitating (or activate or assist in activating) a latent (dormant) pathogenic microbe *in vivo* thereby to potentiate adjunctive antimicrobial therapy. The adjunctive antimicrobial therapies for use in such applications are those which depend for full efficacy on a non-latent or active (e.g. growing or replicating) target pathogen population (for example adjunctive therapies based on certain types of antibiotic). Thus, the materials of the invention may act synergistically with various antimicrobial compounds in antimicrobial therapy.

In a preferred embodiment, the invention is used to potentiate the antimicrobial therapy of tuberculosis, for example involving co-administration of one or more of isoniazid, rifampicin, pyrzinamide and/or ethambutol (or streptomycin).

Particularly useful materials for use in such therapies include for example the RP-factors of the invention, RP-factor agonists, activators and mimetics. Such agonists, activators or mimetics may comprise: the RP-factor receptor antibodies as hereinbefore described; the RP-factor convertase as hereinbefore defined; an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or operably coupled combinations thereof.

The RP-factor receptor antibodies for use in such methods are those which serve to trigger an efferent signal transduction pathway at the RP-factor receptor. They may therefore act as RP-factor mimetics, breaking latency/dormancy and acting to resuscitate the pathogen.

Particularly useful in such methods are mutant RP-factors having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified). Such mutant RP-factors may be active against a broad range of pathogens (e.g. against substantially all pathogenic or infective mycobacteria) or targeted against specific pathogens (for example, *M. tuberculosis* and *M. leprae*).

The antibodies, RP-factors, receptors and convertases discussed above may be administered directly or *via* a live vaccine vehicle. Such live vaccines vehicles comprise microorganisms which have been genetically engineered to express (and preferably secrete) the therapeutically active antibodies, RP-factors, receptors and convertases of the invention *in vivo*.

The invention therefore finds application in the treatment of a wide variety of microbial infections, and finds particular application in the treatment of latent microbial (e.g. bacterial) infections.

In preferred embodiments, the invention finds application in the treatment of actinomycete or mycobacterial infections, for example those involving *M. tuberculosis, M. leprae, M. bovis, M. kansasii* and *M. avium*.

Other infections which may be treated according to the invention include those involving *Corynebacterium* spp. (including *Corynebacterium diphtheriae*), *Tropheryma whippelii*, *Nocardia* spp. (including *Nocardia asteroides* and *Nocardia brasiliensis*), *Streptomyces* spp. (including *Streptomyces griseus, Streptomyces paraguayensis* and *Streptomyces somaliensis*), *Actinomadura* spp., *Nocardiopsis* spp., *Rhodococcus* spp., *Gordona* spp., *Tsukamurella* spp. and *Oerskovia* spp. as well as other pathogenic organisms from the group referred to as high G+C Gram-positive bacteria. Other infections which may be treated include those involving pathogenic low G+C Gram-positive bacteria (e.g. *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp. and *Lactobacillus* spp.).

The invention may also be embodied in various vaccines or immunotherapeutic agents.

Such vaccines or agents target one or more elements of the RP-factor mediated signal transduction pathway described herein (and in particular, the RP-factor or RP-factor receptors/convertases themselves). Thus, the RP-factors may be administered as part of a vaccine or immunotherapeutic composition to elicit an immune response directed against endogenous RP-factor in the patient, so reducing, preventing activation of the pathogen and so slowing or halting the progression of the infection.

Alternatively (or in addition), the RP-factor receptors/convertases may be administered as part of a vaccine or immunotherapeutic composition to elicit an immune response directed against receptors for pathogen-borne RP-factor in the patient. In this way, cellular and/or humoral immune responses may be stimulated against the pathogen(s) and/or activation of a latent pathogen (or its continued growth or multiplication) *via* the RP-factor signal transduction pathway may be reduced or prevented, so slowing or halting the progression of the infection.

The invention also finds application in the preparation of live vaccines: attenuated microbial strains can be constructed in which the gene(s) encoding (or regulating the expression or activity of) one or more RP-factors are mutated. Such attenuated vaccines may be based on mutant strains of actinomycetes, mycobacteria (for example *M. tuberculosis, M. leprae, M. bovis* (such as *M. bovis* BCG), *M. kanasii* and *M. avium*), *Corynebacterium* spp. (including *Corynebacterium diphtheriae*), *Tropheryma whippelii, Nocardia* spp. (including *Nocardia asteroides* and *Nocardia brasiliensis*), *Streptomyces* spp. (including *Streptomyces griseus, Streptomyces paraguayensis* and *Streptomyces somaliensis*), *Actinomadura* spp., *Nocardiopsis* spp., *Rhodococcus* spp., *Gordona* spp., *Tsukamurella* spp. and *Oerskovia* spp. as well as other pathogenic organisms from the group referred to as high G+C Gram-positive bacteria.

Particularly useful in such attenuated vaccines are strains bearing mutated RP-factor-encoding genes. Such mutations may be frameshift, deletion, insertion and/or substitution mutations. In preferred embodiments the mutations are null mutations (e.g. non-reverting null mutations), and may prevent growth of the microbe (i.e. "attenuate" it). In other embodiments the mutations may result in the expression of mutant RP-factors having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified) and/or which lack a functional signalling domain. Such mutant RP-factors may bind with high affinity (and/or essentially irreversibly) and non-productively to the RP-factor receptors/convertases and so block RP-factor-ligand binding or RP-factor maturation. The attenuated microbial strains of the invention may also bear mutations in other genes (for example, in other genes essential to growth), and may also bear one or more genetic marker elements.

Biotechnological applications

It is widely recognised that the great majority (probably well in excess of 99%) of soil organisms have not yet been cultured. Hitherto uncultured organisms are also expected to exist in other sources. The present invention may be used to permit the recovery of

such organisms by culture from any source. Thus, the invention provides a way of unlocking an immense reservoir of biodiversity that is known to exist, but is presently inaccessible.

Thus, the present invention provides an unprecedented resource from which libraries of potentially useful microorganisms and biomolecules can be generated. Such libraries can then be used in screening methods to search for medically or industrially useful products.

Thus, in another aspect the invention provides a process for producing a library of biomolecules comprising the steps of: (a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived); (b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture; and (c) isolating microorganisms from the culture of step (b).

The process may further comprise the step of screening the isolated microorganisms for those which elaborate one or more biomolecules of interest (for example a metabolite, enzyme, antibiotic (e.g. antiviral, antibacterial or antifungal agent) or toxin).

Also contemplated is a biomolecule produced by (or obtainable by) the above process, or a derivative thereof.

In another aspect, the invention provides a process for producing a library of microorganisms (e.g. bacteria) comprising the steps of: (a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived sample); (b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture; (c) isolating microorganisms from the culture of step (b).

Also contemplated is a microbe produced by (or obtainable by) the above process, or a derivative (e.g. mutant) thereof.

Exemplification

The invention will now be described in more detail with reference to several Examples. These are for exemplary purposes only and are not intended to limit the invention in any way.

Explanation of the Figures

Figure 1: Part A. Multiple sequence alignment of the predicted amino-acid sequences of RP-factor-like gene products from *M. luteus*, *M. tuberculosis*, *M. leprae* and *Streptomyces coelicolor*. Proteins similar to the RP-factor are derived from *M. tuberculosis* (accession nos. U38939,

nt 2406-2765, and Z81368, nt 33932-34396) and *M. leprae* (accession nos. L01095, nt 12292-12759, and L04666, nt 25446-24921). The DNA sequences of interest in accession Z81368 are also encompassed by accession AD000010. N-terminal residues corresponding to predicted Gram-positive signal sequences are underlined. The *M. leprae* L04666 sequence may also contain a short, 32 aa signal peptide.

Part B. Multiple sequence alignment of gene products related to YabE of *Bacillus subtilis*. The alignment is given in two parts (A and B), with aligned residues in upper case. Those residues which are conserved (or conservatively substituted) in two or more sequences are in bold. In Part A, perfectly conserved residues are marked with a hash (#) and conservative substitutions with a dot (.). Cperfring is an incomplete ORF1 from *Clostridium perfringens* (Acc. No. U04966); Caceto506 is an incomplete ORF from contig 506, *Clostridium acetobutylicum* genome sequencing project. YocH from *B. subtilis* and YabE from *B. subtilis* are YocH and YabE predicted gene products from the *B. subtilis* genome sequencing project (Acc. Nos. BG13521 and P37456).

Part C. Alignment of the RP-factor C-terminal domain with known and hypothetical wall-associated proteins from other organisms. Perfectly conserved residues are marked with an asterisk, those conserved in at least 7 sequences are marked with a dot (.).

Part D. Motifs in the C-terminus (residues 158-322) of MtubMTV043.

Part E. Alignment between the predicted amino acid sequence of the *M. luteus* RP-factor and p60 proteins from *Listeria* spp. Many of the residues that are conserved in the alignment between the C-terminal portion of the *M. luteus* RP-factor (residues 125-220) and the *L. monocytogenes* EGD p60 protein (residues 158-245), are also conserved in the p60 protein from six other *Listeria* spp.

Figure 2: Part A. The sequence of the RP-factor-encoding gene and its predicted product. The nucleotide sequence is in lower case with PCR primers in bold. The predicted protein sequence is in upper case bold (single letter code). Protein and peptide microsequence data used for oligonucleotide design are in upper case italics.

Part B. The sequence of a 299 base pair DNA fragment encoding part of an RP-factor from *Streptomyces coelicolor*. The deduced amino acid sequence is given below the DNA sequence using the single letter amino acid code.

Figure 3: The elution profile of the resuscitation activity. Fractions eluted from the DEAE-sepharose column (see Materials and Methods) with 0.25 M KCl were applied to a Mono Q column which was developed with a 20ml linear gradient from 0.08 to 0.28 M KCl in 10 mM Tris-Cl buffer supplemented by 10% glycerol, pH 7.4. 10 ml of a diluted

suspension of starved cells (CFU 3.10^6cells.ml^{-1}, total count $1.2.10^9$cells.ml^{-1}) were added to 200 ml of LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract containing of 2 µl of each fraction in 5-10 replicates in the Bioscreen instrument. For details see Materials and Methods. **A:** absorbance at 280nm and magnitude of KCl concentration. **B:** resuscitation activity. **C:** SDS-PAGE profile of the fractions following DEAE-cellulose and Mono Q chromatography. Lanes : 1, markers (94,000, 67,000, 43,000, 30,000, 20,100, 14,400); 2, fraction from DEAE-cellulose column; 3, purifed preparation (fraction number 8 from the Mono Q -column). **D:** Reduction of apparent lag phase of viable cells. 10 µl of a diluted suspension of viable, stationary phase cells (viable count 20 cells) was added to 200 µl of LMM supplemented with 0.5 % w/v L-lactate and containing 2 µl of each fraction (from a different experiment to that shown in parts A and B) in 5-10 replicates in the Bioscreen instrument. The apparent lag phase was estimated by extrapolating the exponential growth line to the abscissa.

Figure 4: Effect of purified RP-factor on *M. luteus*.

A. Concentration dependence of RP-factor activity for resuscitation: resuscitation of dormant cells with different concentrations of RP-factor. 10 µl of a diluted suspension of starved cells (CFU 3.10^6 cells.ml^{-1}, total count 5.10^9 cells.ml^{-1}) was added to 200 µl of LMM supplemented with 0.5 % w/v L-lactate, 0.05% yeast extract and RP-factor in concentrations shown in 5-10 replicates in the Bioscreen instrument. For details see Materials and Methods.

B. Growth of washed cells. Stationary phase cells of *M. luteus* grown in LMM were washed five times by suspension and centrifugation in LMM from which lactate had been omitted. Bacteria were finally suspended in the same medium by repeatedly passing them through a syringe, diluted and inoculated into a 20 ml flask containing LMM or LMM plus 31 pM RP-factor. The initial cell density was 250 viable cells per ml and incubation was at 30°C with intensive shaking. Growth was monitored by plating 0.1ml samples on plates containing broth E solidified with agar.

Figure 5: Detection of RP-factor-like genes in *Micrococcus luteus*, *Mycobacterium smegmatis* and *Streptomyces rimosus*.

	Part A		Part B		Part C	
	M. luteus		*M. luteus*			
	Lane 1	*λBst*EII	*λPst*I		*λPst*I	
	Lane 2	*Cla*I	*Xho*I		*S. rimosus Xho*I	
	Lane 3	*Sal*I	*Stu*I		*S. rimosus Stu*I	
	Lane 4	*Sac*II	*Sma*I		*S. rimosus Sma*I	
	Lane 5	*Pst*I	*Pvu*II		*S. rimosus Pvu*II	
	Lane 6	*Nco*I	*Pst*I		*S. rimosus Pst*I	
	Lane 7	*Nhe*I	*Kpn*I		*S. rimosus Bam*HI	
	Lane 8	*Mlu*I	*Bam*HI		*M. smegmatis Xho*I	
	Lane 9	*Aat*II	*λPvu*II		*M. smegmatis Stu*I	

Lane 10	*λPst*I	*M. smegmatis Sma*I
Lane 11		*M. smegmatis Pvu*II
Lane 12		*M. smegmatis Pst*I
Lane 13		*M. smegmatis Bam*HI
Lane 14		*λPvu*II

Figure 6 : Effect of *M. luteus* RP-factor on the growth of *Mycobacterium smegmatis* (A) and *Mycobacterium bovis* (B) in batch culture as observed turbidimetrically. *M. smegmatis* was grown in broth E, to which was added RP-factor at 31 pMol/L. Cells were inoculated at a level of *circa* 200 per well, and growth was monitored in the Bioscreen instrument. *M. bovis* was grown in Sauton medium, as described in the Materials and Methods section, to which RP-factor (620 pMol/L) was either added or not. The inoculum was *circa* 1.10^5 cells.ml^{-1}, and the OD shown is the average of 10 separate determinations of 10 separate tubes.

Figure 7: A: Purification of His-tagged RP-factor. RP-factor was expressed in *E. coli* HSM174(DE3) and purified as described *infra*. Shown is the SDS-PAGE profile of fractions following Ni^{2+}-chelation chromatography. The molecular weight (kDal) markers (SIGMA) were bovine serum albumin (67), ovalbumin (43), glyceraldehyde 3-phosphate dehydrogenase (36), carbonic anhydrase (30), soya bean trypsin inhibitor (20.1), and lactalbumin (14.4). Lane: 1, markers; 2, crude extract from *E. coli* containing pET19b vector; 3, crude extract from *E. coli* containing pRPF1; 4, purified recombinant RP-factor.

B: Reduction of the apparent lag phase of viable cells of *M. luteus* by purified recombinant RP-factor. For experimental details see the legend for Figure 3C. A dilution factor of 10^0 corresponds to 33 μg RP-factor/ml.

C: Stimulation of the growth of washed cells of *M. luteus* by purified recombinant RP-factor. Stationary phase cells of *M. luteus* grown in LMM were washed 5 times by suspension and centrifugation in LMM from which lactate had been omitted. Bacteria were finally suspended in the same medium by repeatedly passing them through a syringe, diluted, and inoculated into a 20 ml flask with LMM or LMM in the presence of RP-factor (230 pMol/L). The initial cell density was ca. 10^2 viable cells per ml and incubation was at 30°C with intensive shaking. Growth was monitored by plating 0.1 ml samples on plates containing nutrient broth E solidified with agar.

Figure 8: A: Anti-RP-factor serum inhibits the growth of *Micrococcus luteus*. Bacteria were inoculated at an initial density of $5x10^5$ per ml into lactate minimal medium (LMM) and the OD_{600nm} was monitored at intervals. Growth of the cultures was monitored over 140 hours at intervals. The samples labelled LMM + Ab and LMM + control Ab contain equivalent amounts of immune and pre-immune serum, respectively. Immune serum (Ab) and pre-immune serum (control Ab) were employed at a 1:1000 dilution.

B: RP-factor overcomes the inhibitory effect of anti-RP-factor serum on growth of *Micrococcus luteus*. Bacteria were inoculated at an initial density of 10^7 cells per ml and growth was monitored by measuring the OD_{600nm} at intervals. Immune serum

(Ab) and pre-immune serum (control Ab) were employed at a 1:1000 dilution and RP-factor was added at a final concentration of 50 ng/ml.

Figure 9: Part A. Blocked alignment of nine RP-factors (as explained *infra*, MtubZ94752 may be a cognate receptor). Areas of sequence identity/similarity are indicated by the shaded areas. The *S. coelicolor* gene product shown is a fragment.

Part B. Schematic showing the domain structure of some gene products in the RP-factor family.

Figure 10: Effect of recombinant RP-factor on growth of *M. tuberculosis* in Sauton medium. Sauton medium containing 0.05% Tween-80 and 100μMol/L Na oleate + 10% (v/v) supplement (which contains, per litre, 50g bovine serum albumin, 20g glucose, 8.5g NaCl) was inoculated to an initial cell density of 31×10^3 cfu/ml (viable count determined by plating on agar-solidified Middlebrook 7H9 medium containing 10% v/v supplement, composition as detailed above) [total count by microscopy = 10^6 cells per ml] with a 2.5 month-old culture of *M. tuberculosis* strain H37Ra grown in the same medium. Growth of tube cultures at 37°C was measured by determining the OD_{600nm} at intervals for 28 days. The undiluted concentrations of the RP-factors, Rpf (*M.luteus*) and Rpf2 (*M. tuberculosis*), employed for these experiments were ca. 10μg/ml.

Examples

Material And Methods

Organisms and media.

Micrococcus luteus NCIMB 13267 (previously described as "Fleming strain 2665") was grown aerobically at 30°C in shake flasks in lactate minimal medium (LMM) containing L-lactate as described previously. When the culture had reached stationary phase agitation was continued at 30°C for up to 2 months. Cultures were then held aerobically at room temperature without agitation for period for up to a further 2-3 months. The apparent initial viability of these cultures at this point (measured by comparing the plate count with the microscopic count) was less than 10^{-3}.

Mycobacterium smegmatis ("fast" strain, All-Russia State Institute for Contol of Veterinary Preparations, Moscow) was grown in either Sauton medium or nutrient broth E (LabM). Overnight pre-cultures were used to inoculate cultures to an initial density of 10^3 cells/ml. *Mycobacterium bovis* (BCG), *Mycobacterium tuberculosis* H37RV and *Mycobacterium avium* were grown in Sauton medium.

M. luteus *Spent medium preparation.*

Supernatant was obtained after the centrifugation of late logarithmic phase *M. luteus* cultures (200-1000 ml) grown in lactate minimal medium or in the same medium in which lactate was replaced by succinate plus 0.01% yeast extract from which macromolecules had been removed by dialysis. The inoculum consisted of 2% of cells grown in rich medium (Broth E, LabM) and then washed in LMM lacking lactate. The supernatants were passed through a 0.22 μm filter (Whatman) before use.

M. luteus *Cell viability by plating.*

Plates consisting of 1.3% Nutrient Broth E (LabM) or lactate minimal medium were used. Cell dilutions were made in quadruplicate with centrifuged and autoclaved spent medium taken from the starved culture. Plates were incubated at 30°C for 3-5 d.

M. luteus *Cell viability by MPN.*

The MPN assay was performed in a Bioscreen C optical growth analyzer (Labsystems, Finland) using lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract as a resuscitation medium. Dilutions of starved cells were made as described. 10 μl of each dilution (5-10 replicates) were added to a well containing 200 μl of either lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract or the same medium with fraction tested (2-20 μl). Growth (optical density) was monitored using a 600 nm filter. Plates were incubated at 30°C with intensive continuous shaking. The overall measurement period was 120h, each well being measured hourly. The fractions obtained after chromatography were dialysed against elution buffer 2 (see below), diluted in resuscitation medium in various proportions (1:10, 1:100, 1:500, 1:1000, 1:5,000, 1:10,000) and filtered through 0.22 μm Gelman filters before testing. The calculation of the MPN was based on published Tables.

Total cell counts

Unstained cells were counted with a phase-contrast microscope and an improved Neubauer counting chamber. In long-term experiments with mycobacteria, organisms were stained with Ziehl-Neelsen reagent before counting.

Chromatography

Pre-wetted DEAE cellulose was added to culture supernatant (1:10 v/v) and incubated at 4°C for 1h with slow stirring. The cellulose was loaded into a column, and washed with 5 volumes of buffer 1 consisting of 10mM Tris-Cl, 1mM EDTA, 1mM DTT, 10% (v/v) glycerol, pH 7.4 with 10mM KCl. The column was eluted stepwise with 2-3 bed volumes of 0.3M KCl in buffer 1. The fraction obtained was slowly diluted with buffer 1 on ice to give a final KCl concentration of 0.08M. Forty column volumes of this fraction was then loaded onto a DEAE-sepharose fast flow column (1 part of sepharose pre-equilibrated

with buffer 1 containing 0.08M KCl). The column was washed with 5 bed volumes buffer 1 containing 0.08M KCl and eluted stepwise with 3 volumes of 0.25M KCl in buffer 1. The fraction obtained was again slowly diluted with buffer 1 on ice to a final KCl concentration of 0.08M, filtered through a 0.22 μm Gelman filter and loaded onto a Mono Q column (model HR5/5, pre-packed, Pharmacia) equilibrated with buffer 2 consisting of 10mM Tris-Cl, 10% glycerol, pH 7.4 containing 0.08M KCl. The Mono Q column was eluted by a linear gradient from 0.08 M to 0.28 M KCl in buffer 2 (the total volume of the elution was 20 ml). The flow rate and fraction size were 1 ml/min and 1ml/tube respectively. All manipulations except the Mono Q chromatography step were performed at 4°C. The fractions obtained were dialysed against 10 mM Tris-Cl containing 10% glycerol (dialysis is important for the retention of activity) and stored at 4°C for up to 5 days without loss of activity. For prolonged storage in a deep freeze, fractions were dialysed in the same way and glycerol added to a final concentration of 20-30% w/v. The protein content in purified preparations was estimated by tryptophan fluorescence using lysozyme as a standard.

Trypsin treatment:

Trypsin was added to the active, dialysed fraction obtained from the mono Q column and diluted by LMM supplemented with 0.5 % w/v lactate and 0.05% yeast extract (1:100) (the final concentration of trypsin was 50 ug/ml). The mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of trypsin inhibitor (100 ug/ml). In control experiments trypsin inhibitor was added to the mixture (100 ug/ml) prior to incubation.

PAGE electrophoresis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli. Chromatographic fractions were dialysed against 10mM Tris HCl, pH 7.4 for 4-5 h, dried in a speed-vacuum apparatus (1.5h), dissolved in sample buffer (Sigma, S-3401), loaded onto 15% acrylamide gel and run at a constant voltage of 200V . The gel was stained with colloidal Coomassie G (Sigma).

Chemicals.

Nutrient Broth E, yeast extract and agar were obtained from Lab M, whilst L-lactate (Li salt), succinate, trypsin, soybean trypsin inhibitor and DEAE-Sepharose fast flow were obtained from Sigma. DEAE cellulose DE52 was obtained from Whatman, and Mono S and Mono Q from Pharmacia. Other chemicals were of analytical grade and were obtained from Sigma or BDH.

DNA manipulations.

Protein microsequence data from the N-terminus (**ATVDTWDRLAE**exSNGTxD) and an internal peptide (**VGGEGYPHQASK**) obtained from the purified RP-factor were used to

design two oligonucleotides, denoted A1 [GCSACSGTSGACACSTGGGACCGSCTSGCSGAG] and A2 [GCYTGRTGIGGRTAICCYTCICC], respectively. Taq polymerase was employed under standard conditions to amplify a 147 bp PCR product from *M. luteus* DNA with these primers. The PCR product obtained from *M. luteus* DNA with these two primers was labelled with digoxygenin and used as a probe for Southern hybridisation experiments. *Sma*I-digested genomic DNA was size-fractionated by agarose gel electrophoresis and circa 1.4 kbp fragments were cloned in pMTL20 and established in *E. coli* strain DH5α. Two recombinant plasmids carrying the desired insert were detected by hybridisation, confirmed by PCR using oligonulceotides A1 and A2, and one of them was manually sequenced on both strands using the dideoxy chain termination method.

Standard procedures were employed to isolate DNA from *M. luteus* and *M. smegmatis*. *Streptomyces rimosus* DNA was kindly supplied by Dr. D. Hranueli. Southern hybridisations with *M. smegmatis* and *S. rimosus* DNA were initially carried out under non-stringent conditions (0.5 SSC, 37°C). Stringent conditions (0.1 SSC, 65°C) were subsequently employed for screening an ordered cosmid library of *Streptomyces coelicolor* A3(2) DNA.

Purification of RP-factor

RP-factor purified from culture supernatants of cells grown in lactate minimal medium, according to the protocol described in Materials and Methods, revealed the presence of a significant amount of polymeric material eluted from all types of columns used, which inhibited both the resuscitation of dormant cells and the growth of viable cells of *M. luteus*. Moreover, elevated concentrations of this material could even cause the lysis of cells (not shown). This inhibitory material appears to be a polymer derived from lactate, as lactate-containing LMM stored for 10 hours at room temperature without cells and subjected to the same procedure of purification revealed inhibitory properties similar to those of this spent medium. To avoid this problem we replaced lactate in the growth medium with succinate, although for good growth it proved necessary to add a small amount (0.01 % w/v) of yeast extract dialysed to remove macromolecules.

Using succinate-grown cultures, the active fraction was purified by a combination of anion exchange media (see Material and Methods). The final activity was eluted at around 180 mM KCl from a linear KCl gradient (from 0.08 to 0.28M KCl) on a MonoQ column in 3 adjacent fractions (Fig. 3). It is worth mentioning that it proved important to dialyse the fractions before testing their activity because some fractions were inactive before dialysis. Active fractions did not change their resuscitation activity after dilution up to 400 times (v/v).

Interestingly, those fractions which were active in causing resuscitation could also increase the growth rate of viable cells.

The resuscitation-promoting material from the final purification step was checked by

SDS-PAGE. The final product (Fig. 3C) proved to consist of a single protein with a molecular weight estimated to be ca 16kD. All active fractions consist of single band with maximum content of protein in fraction N9.

Cloning of the RP-factor gene

Two primers were designed from protein microsequence data obtained for the N-terminus of the purified RP-factor and for an internal peptide. They were used to amplify a 147 bp fragment of *M. luteus* DNA, which was cloned and sequenced. The complete gene was then obtained by a combination of inverse PCR using oligonucleotides G1 and G2 and isolation of a 1.4 kbp *Sma*I genomic restriction fragment. Sequencing revealed that the original PCR product was part of a gene capable of encoding a protein having a signal sequence (Fig. 2A). The predicted size of the secreted form of the gene product is 19,148 Dal, and its predicted N-terminal amino acid sequence agrees with the protein microsequence data, including residues that were not used in primer design (Fig. 2A). The fact that the predicted gene product is larger than the RP-factor purified from culture supernatants suggests that it may, for example, be secreted as a precursor which is converted to its biologically active form upon contact with its cognate receptor/convertase.

Identification of RP-factor homologues

A BLAST search was undertaken using the predicted amino acid sequence of the ORF from *M. luteus* as query. Seven genes with substantial similarity have been sequenced previously. Five are found in *M. tuberculosis* and two in *Mycobacterium leprae* (Fig. 1A). One or more gene products in each organism appear to have a secretory signal sequence (underlined in Fig. 1A). The functions of the predicted products of these mycobacterial genes are unknown; they were found by genome sequencing projects. The BLAST search also revealed similarity between residues 126-220 of the RP-factor and a conserved segment of the (major extracellular) p60 proteins that have been implicated in adherence of *Listeria* spp. to 3T6 mouse fibroblasts suggesting, perhaps, a possible role for the RP-factor or a proteolytic product thereof in adhesion in *M. luteus* (Fig. 1E).

In common with *M tuberculosis* and *M. leprae*, *M. luteus* contains a second gene similar to that encoding the RP-factor. Southern hybridisation experiments, using DNA samples cleaved with a range of different restriction enzymes, and the cloned 147 bp fragment as probe (Figs. 5A & B), reveal two hybridising bands. The stronger hybridisation signal arises from the gene encoding the secreted RP-factor. The other gene may correspond to one of the other mycobacterial genes identified above.

Southern hybridisation experiments, using the 147 bp fragment as probe, as well as PCR experiments, using two oligonucleotides based on highly conserved amino acid motifs as primers, indicate that genes encoding proteins similar to the RP-factor are of widespread occurrence, at least throughout Gram-positive bacteria whose DNA has a high G+C content. Similar genes are detectable by either or both of these methods in all six

Streptomyces species we have tested, including *Streptomyces rimosus* (Fig. 5C) as well as in other mycobacteria, including *Mycobacterium smegmatis* (four similar genes - Fig. 5C), *Mycobacterium bovis* (BCG) and *Corynebacterium glutamicum* (2 similar genes).

Domain structure

The sequence information shows that the RP-factor gene and all of its mycobacterial homologues share a secretory signal sequence and a particularly highly conserved, ca. 70-residue segment. One (MTubZ94752) also has a membrane anchoring motif. The conserved 70-residue segment is a candidate for a signalling domain. Most of this segment is weakly hydrophilic (Kyte-Doolittle) and is predicted to form amphipathic α-helical (Garnier-Robson; Chou-Fasman) or β-sheet regions (Eisenberg). Overall, the segment has a low surface probability (Emini). The C-terminal section, by contrast, is much less highly conserved and might be considered a better candidate for determining localization or specificity (i.e. be a cellular compartment-targeting or specificity-determining domain). By analogy with other protein signalling systems (e.g. many pro-hormones in animals, and systemin in plants) it is possible that the proximate signalling molecule is a proteolytically cleaved product.

Two acidic residues, D7 & E13 (numbering according to the *M. luteus* secreted protein), within this segment are absolutely conserved. The KAEQIKRAE segment (residues 51-59) represents an island of particularly high surface probability. These elements may form part of functional domains within the RP-factor protein.

The conserved domain contains four conserved tryptophan residues (one of which is in a region of high surface probability DTWDR - residues 4-8). In the complex between human growth hormone and its first bound receptor, interactions involving two surface-located tryptophan residues in the receptor account for more than 75% of the binding free energy of the complex (Clackson and Wells, Science 267, 383-386, 1995). The two conserved cysteine residues may form a disulphide bridge.

Alignments showing the domain structures of the various proteins are shown in Figs.9A and 9B.

RP-factor activity

As well as resuscitating dormant cells, the purified RP-factor from *M. luteus* has been tested for growth-stimulatory activity against *M. luteus* and several other organisms. It strongly stimulates the growth *of M. luteus* and *M. smegmatis* and it appears to have weaker activity on *M. tuberculosis*, *M. bovis* (BCG) and *M. avium* (see Fig. 6). In all cases, there is a shortening of the apparent lag phase in batch culture (see Figs. 3D, 4B, 6B and Table 1). The factor is active in poor media and in poor media supplemented with yeast extract and it loses activity after boiling or treatment with trypsin.

When ca. 40 pMol/L RP-factor was added to washed cells of *Mycobacterium smegmatis*,

growth occurred after 20-24 hr, whereas the control lacking RP-factor showed no growth after 6 days. Experiments with slowly growing mycobacteria yielded similar results. Growth of *M. bovis* (BCG) was also strongly stimulated by 40 pMol/L RP-factor: growth occurred after 14 days whereas the control lacking RP-factor showed no growth after 90 days. Finally, RP-factor also stimulated the growth of *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, *Mycobacterium avium* and *Mycobacterium kansasii* (see Table 1).

Table 1. Purified *M. luteus* RP-factor stimulates growth of mycobacteria

Organism	Bacterial growth[$] RP-factor omitted	RP-factor added
Mycobacterium tuberculosis H37Ra	1.3 ± 1.9 (5)	110 ± 32 (5)
Mycobacterium tuberculosis H37Rv	1.5 ± 2 (4)	45 ± 28 (4)
Mycobacterium avium	0 (3)	>300 (3)
M. bovis (BCG)	0 (5)	54 ± 38 (5)
*M. smegmatis**	0 (8)	225 ± 44 (8)
Mycobacterium kansasii	2.5 ± 2.5 (3)	90 ± 77 (3)

[$]Growth was estimated microscopically (magnification times 600) after 14 days of incubation; ca. 50 µl of each culture was fixed, stained using Ziehl-Neelsen reagent and counted. Values in the body of the Table are average numbers of cells in a microscope field (10-20 fields counted) ± standard deviation with the number of determinations in parentheses. RP-factor (after elution from the Mono Q column and dialysis) was used at a concentration of circa 40 pMol/L; activity was lost after either trypsin treatment, heating (autoclaving) or filtration through a 12 kDal cutoff membrane.
*Washed cells of *M. smegmatis* were used for this experiment.

Isolation and characterisation of the gene encoding the second homologue from *M. luteus*

A combination of inverse PCR using oligos G1 and G2 (see Fig. 2A) as primers, and cloning of suitably sized genomic restriction fragments, can be employed to isolate the gene encoding the second homologue from *M. luteus*. The sequence of the gene can then be determined, taking care to eliminate any possible PCR errors by analysis of genomic clones and direct sequencing of PCR fragments obtained by combining the products of multiple, independent PCR reactions. Comparative sequence analyses of the proteins from *M. luteus*, *M. leprae* and *M. tuberculosis* can then be used to refine predictions concerning residues, sequence motifs and structural motifs which may be important for biological function.

Over-expression and purification of *M. luteus* and *M. tuberculosis* gene products in *E. coli*

PCR primers can be designed, incorporating suitable restriction sites such that sequences encoding the secreted forms of the *M. luteus* and the *M. tuberculosis* RP-factors can be amplified and inserted, in the correct reading frame, into commercially available plasmids (pET or pCAL vectors). The PCR-amplified fragments can first be cloned in a pBluescript KS II vector (Stratagene) so that their entire sequence can be verified, to eliminate possible PCR errors. (This material can also be employed for site-directed mutagenesis - *vide infra*.) The pET or pCAL constructs can then be employed to obtain controlled expression of large quantities of histidine- or calmodulin binding peptide-tagged proteins that can be purified, essentially to homogeneity, in a single step. Finally, the tags used in protein purification can be removed (using enterokinase or thrombin, as appropriate).

Expression of RP-factor from *Micrococcus luteus* in *E. coli*

Two primers [5'-GTCAGAATTCATATGGCCACCGTGGACACCTGGG-3'] and [5'-TGACGGATCCTATTAGGCCTGCGGCAGGACGAG-3'] were employed to amplify (5 cycles of 30s at 94°C, 30s at 60°C, 30s at 72°C, followed by 15 cycles of 30s at 94°C, 60s at 72°C) the RP-factor coding sequence (i.e. lacking the signal sequence) from the cloned 1.4 kbp *Sma*I fragment of genomic DNA. It was first established in *E. coli* DH5α as a 567 bp *Eco*RI-*Bam*HI fragment in pMTL20 and then excised as a 562 bp *Nde*I - *Bam*HI fragment, inserted into pET19b (Novagen) and re-established in *E. coli* DH5α. The sequence of the PCR product and vector-insert junction in this plasmid, denoted pRPF1, was verified. RP-factor was expressed from RPF1 after transforming it into *E. coli* HSM174(DE3). The protein, containing a His_{10}-tag at the N-terminus, was isolated by sonicating bacteria, previously grown to an OD_{600nm}=0.6 and induced with 0.4 mM IPTG for 4 h, in a modified binding buffer (MBB - 5mM imidazole pH7.9/0.5M NaCl/20mM Tris-HCl/8M urea) containing 5 mM DTT and 2 mM EDTA. After low speed centrifugation, low MW compounds, including EDTA and DTT, were removed by elution through a Sephadex G10 column pre-equilibrated with MBB. A Ni^{2+}-chelation column (Ni^{2+}-coordinated iminodiacetic acid immobilized on Sepharose 6B), was loaded with the G10 eluate, washed with 20 vol MBB and then successively eluted with four 10 vol aliquots of MBB containing 0.01 M, 0.05 M, 0.2 M and 1 M imidazole, respectively. The column was finally eluted with strip buffer (20 mM Tris-HCl, pH 7.9/100 mM EDTA/0.5 M NaCl). Monoclonal anti-(polyHis) antibodies (Sigma, clone His-1) were employed for immunoblot analysis of fractions subjected to SDS PAGE electrophoresis and electroblotted using standard methods. Fractions were dialysed against buffer 2 and assayed for biological activity as indicated above.

Analysis of recombinant RP-factor

The coding sequence corresponding to the secreted form of RP-factor, starting at residue A_{39}, was inserted into pET19b to generate plasmid pRPF1 (*vide infra*). Extracts of IPTG-induced *E. coli* strain HSM174(DE3) containing pRPF1 were challenged with a poly-His

antibody. A strong signal was associated with a protein (apparent size 29 kDal, predicted size 22 kDal) which was eluted from the affinity column by 1M imidazole (Fig. 7A). The His-tagged protein from HSM174(DE3) reduced the apparent lag phase of viable cells of *M. luteus*, whereas the control (material eluted from the same column under the same conditions when an extract from cells containing plasmid vector only was applied) showed no activity (Fig. 7B). The association of biological activity with the recombinant protein, produced in *E. coli* containing pRPF, and the absence of biological activity in the isogenic control containing pET19b, demonstrates unequivocally that the active molecule is indeed a product of the *rpf* gene.

Antibody preparation

A rabbit was immunized three times at one week intervals using recombinant RP-factor (the recombinant protein prepared as described above). The protein was administered at 300 μg of protein per injection in incomplete Freud's adjuvant (0.5 ml protein and 0.5 ml adjuvant) Blood was collected before administration was started and on the 11th day after the last injection. The immunoglobulin fraction was obtained by standard procedures using PEG. Antibodies were additionally purified on a protein G-superose column according to the standard (Pharmacia) protocol. The final protein concentration was adjusted spectrophotometrically to 1 mg/ml.

Alternatively, monoclonal antibodies can be produced using established techniques.

Use of anti-RP-factor antibody to inhibit bacterial growth

Micrococcus luteus was inoculated at an initial density of 5×10^5 per ml into lactate minimal medium (LMM) and the OD_{600nm} was monitored at intervals. Growth of the cultures was monitored over 140 hours, and the presence of the anti-RP-factor serum (prepared as described above under "Antibody preparation") completely inhibited bacterial growth (see Figure 8).

Expression of a *M. tuberculosis* RP-factor in *E. coli*

Two primers [5'-ATCAGAATTCATATGGACGACATCGATTGGGACGC-3'] and [5'-CGCAGGATCCCCTCAATCGTCCCTGCTCC-3'] were employed to amplify (5 cycles of 30s at 94°C, 30s at 58°C, 30s at 72°C, followed by 25 cycles of 30s at 94°C, 60s at 72°C) the RP-factor coding sequence (i.e. lacking the signal sequence) from *M. tuberculosis* H37Rv genomic DNA. The PCR product was first established in *E. coli* DH5a as a 336 bp *Eco*RI-*Bam*HI fragment in pMTL20 and then excised as a 331 bp *Nde*I - *Bam*HI fragment, inserted into pET19b (Novagen) and re-established in *E. coli* DH5a. The sequence of the PCR product and vector-insert junction in this plasmid, denoted pRPF2, was verified. The *M. tuberculosis* RP-factor was expressed from pRPF2 after transforming it into *E. coli* HSM174(DE3). The protein, containing a His_{10}-tag at the N-terminus, was isolated by sonicating bacteria, previously grown to an OD_{600nm} = 0.9 and induced with 0.4 mM IPTG for 4 h, in binding buffer (BB - 5mM imidazole pH7.9 / 0.5M

NaCl / 20 mM Tris-HCl / 8M urea). After low speed centrifugation, a Ni^{2+}-chelation column (Ni^{2+}-coordinated iminodiacetic acid immobilised on Sepharose 6B), was loaded with the supernatant, washed with 20 vol BB, 20 vol BB containing 100 mM imidazole, and then eluted with 10 vol BB containing 0.5 M imidazole. Additional purification was achieved by MonoQ column chromatography (*vide infra*, save that the salt gradient was from 0.1 M to 1M NaCl). Monoclonal anti-(polyHis) antibodies (Sigma, clone His-1) were employed for immunoblot analysis of fractions subjected to SDS PAGE electrophoresis and electroblotted using standard methods. Fractions were dialysed against buffer 2 and assayed for biological activity as indicated above.

Analysis of a recombinant *M. tuberculosis* RP-factor

The coding sequence corresponding to the secreted form of the *M. tuberculosis* RP-factor (g1655671; acc. no. Z81368), starting at residue D_{50}, was inserted into pET19b to generate plasmid pRPF2 (*vide infra*). Extracts of IPTG-induced *E. coli* strain HSM174(DE3) containing pRPF2 were challenged with a poly-His antibody. A strong signal was associated with a protein which was eluted from the affinity column by 0.5M imidazole. The histidine-tagged protein from HSM174(DE3) caused a slight but significant enhancement of the growth of *M. tuberculosis* H37Rv, as shown in Fig. 10. It also stimulated the growth of *M. luteus* in LMM. The control culture attained a final OD_{600nm} of 1.0, whereas cultures containing the RP-factor (1:100,000 dilution) attained a final OD_{600nm} of between 2.0 and 6.0.

Effect of *M. luteus* RP-factor on growth of *Mycobacterium tuberculosis* cells isolated from macrophages

In three independent experiments, dormant/latent *M. tuberculosis* cells isolated from cultured murine peritonial macrophages were resuscitated by the *M. luteus* RP-factor. The total number of *M. tuberculosis* cells in the heterogeneous suspension obtained from murine macrophages was determined microscopically. The viable cell count was determined by plating on agar-solidified Sauton medium containing 10% (v/v) supplement (which contains, per litre, 50 g bovine serum albumin, 20g glucose, 8.5g NaCl) or by the MPN method, using liquid Sauton medium containing 10% (v/v) supplement (see above).

The viable count (MPN) of these cell suspensions was enhanced between 25 and 2,500 times by the presence of the *M. luteus* RP-factor (added at a final concentration of 10 ng/ml) (see Table 2). All values in the body of the table are numbers of bacteria per ml suspension

Peritoneal macrophages were obtained from white mice (wild type) by a standard protocol. Infection of macrophages by *M. tuberculosis* "Academiya" (laboratory strain) was performed *in vivo* by intraperitonial injection of 10^6 cells (total count) per mouse followed by incubation for 6 days (1st passage). For the second and third passages macrophage cells in monolayers were infected using *M. tuberculosis* cells isolated from

macrophages from the previous passage.

TABLE 2: Effect of *M. luteus* RP-factor on growth of *Mycobacterium tuberculosis* cells isolated from macrophages

Experiment	Total count [x] (determined microscopically)	Viable count (determined by plating)	Viable count (MPN)	MPN in presence of RP-factor
I	$10^6 > x > 10^5$	90	70	4.10^3
II	$10^6 > x > 10^5$	9	40	1.10^3
III	2.10^6	<1	<1	24.10^3

Macrophages were grown as a monolayer on plastic petri dishes (10^6 cells/5 cm^2) in standard RPMI medium containing gentamicin and penicillin (10μg/ml, each) under standard conditions (CO_2/O_2 mixture in a 37°C incubator). *M. tuberculosis* cells were recovered from macrophages by passing them repeatedly through a thin syringe needle. Macrophage cell debris was removed by low speed centifugation and *M. tuberculosis* cells were then collected by centrifugation at higher speed.

Effect of *yabE* and *yocH* knockout mutations on growth of *Bacillus subtilis*

The entire *yabE* coding region together with flanking sequences was amplified from *B. subtilis* genomic DNA using primers D11 [5'-GAAGAGAATTCCTTCCATCACGA-3'] and D12 [5'-CCAAACGAATTCGGTCAATCAC-3'] as a 1803 bp product. A 1186 bp *Hin*dIII-*Bcl*I fragment encompassing the 3' end of the coding sequence was excised from the PCR product, ligated with *Hin*dIII + *Bam*HI-digested pMTL20, and used to transform *E. coli* strain DH5α with selection for ampicillin-resistance. Plasmid pYABE was isolated from one of the transformants. A 763 bp *Hin*dIII-*Bam*HI fragment from entirely within the *yabE* coding sequence was excised from the pYABE, ligated with *Hin*dIII + *Bam*HI-digested pMUTIN4, an integrating plasmid that may be employed for generating knockout mutations in *B. subtilis* (Edwards & Errington, 1997, Molecular Microbiology, 24, 905-915) and used to transform *E. coli* strain XL1-Blue with selection for ampicillin-resistance. Plasmid pYAB2, containing an internal segment of the *yabE* coding sequence, was isolated from one of the transformants. A 1207 bp *Hin*dIII-*Eco*RI fragment encompassing the 3' end of the *yabE* coding sequence was excised from pYABE, ligated with *Hin*dIII + *Eco*RI digested pMUTIN4 and used to transform *E. coli* strain XL1-Blue

with selection for ampicillin-resistance. Plasmid pYAB3, containing the 3' end of the *yabE* coding sequence, was isolated from one of the transformants.

The entire *yocH* coding region together with flanking sequences was amplified from *B. subtilis* genomic DNA using primers D10 [5'-GCAAGGATCCCAGACTAAAAAAACAG-3'] and D9 [5'-ATCAGGATCCATATTATTAGTTTAAGA-3'] as a 1145 bp product. A 358 bp *Hpa*I fragment from entirely within the *yocH* coding sequence was excised from the PCR product, ligated with *Sma*I-digested pMTL20, and used to transform *E. coli* strain XL1-Blue with selection for ampicillin-resistance. Plasmid pYOC2a, containing an internal segment of the *yocH* coding sequence, was isolated from one of the transformants. The insert in this plasmid was then excised from pYOC2a as a 385 bp *Eco*RI-*Hin*dIII fragment and inserted into pMUTIN4, to yield pYOC2. A 307 bp *Hin*dIII-*Bam*HI fragment encompassing the 3' end of the *yocH* coding sequence was excised from the 1145 bp PCR product, ligated with *Hin*dIII + *Bam*HI digested pMUTIN4, and used to transform *E. coli* strain DH5*α* with selection for ampicillin-resistance. Plasmid pYOC3, containing a DNA segment encompassing the 3' end of the *yocH* coding sequence, was isolated from one of the transformants.

Plasmids pYAB2, pYAB3, pYOC2 and pYOC3 were linearised with *Apa*I, which cleaves once in the pMUTIN4 vector sequences, ligated with T4 DNA ligase and employed to transform *Bacillus subtilis* strain SA253 *nonA nonB leuA8 arg-15* with selection for resistance to erythromycin on a rich nutrient medium (LB + 1 μg Em/ml). Em^R transformants were then picked and verified by Southern hybridization. Using the integrating plasmid as probe, and digesting the chromosomal DNA with *Apa*I, strains harbouring a single copy of the integrated plasmid gave two hybridising bands whereas the wild type (and any spontaneous Em^R mutants that were present) gave a single hybridising band.

Analysis of the products of transformation with each of the four plasmids indicates that *yabE* and *yocH* gene products are required for growth (at least under certain conditions) in *B. subtilis*.

CLAIMS:

1. Isolated RP-factor.

2. The factor of claim 1 which is a secreted RP-factor.

3. The factor of claim 1 which is a non-secreted RP-factor (e.g. a cell-associated or cytosolic factor).

4. The factor of any one of the preceding claims which is derived from a bacterium (e.g. a pathogenic bacterium).

5. The factor of claim 4 which is derived from:
(i) a high G+C Gram-positive bacterium; or
(ii) a low G+C Gram-positive bacterium (for example *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp. and *Lactobacillus* spp.).

6. The factor of claim 5(i) which is derived from:
(a) *Micrococcus* spp. (e.g. *M. luteus*); or
(b) *Mycobacterium* spp. (for example a fast- or slow-growing mycobacterium, e.g. *M. tuberculosis, M. leprae, M. smegmatis* or *M. bovis*); or
(c) *Streptomyces* spp. (e.g. *S. rimosus* and *S. coelicolor*); or
(d) *Corynebacterium* spp. (e.g. *C. glutamicum*).

7. A homologue, derivative, allelic form, species variant, mutein or equivalent of the factor of any one of the preceding claims.

8. A factor of any one of the preceding claims which comprises (or consists of) the RP-factor signalling domain.

9. A factor of any one of the preceding claims which comprises (or consists of) the RP-factor specificity-determining domain.

10. Recombinant RP-factor, wherein the RP-factor is for example as defined in any one of the preceding claims.

11. A pharmaceutical composition (e.g. a vaccine) comprising an RP-factor as an active ingredient (for example the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of the preceding claims), the RP-factor for example being present at a concentration sufficient to confer biological activity on the pharmaceutical composition.

12. An RP-factor (for example the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) or pharmaceutical composition as defined in any one of the

preceding claims) which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

13. An antibody (or antibody derivative) specific for the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of the preceding claims.

14. The antibody of claim 13 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

15. Isolated RP-factor receptor or convertase.

16. The receptor/convertase of claim 15 which is derived from a source as defined in any one of claims 4-6.

17. A homologue, derivative, allelic form, species variant, mutein or equivalent of the receptor/convertase of claim 15 or 16.

18. Recombinant RP-factor receptor/convertase, wherein the receptor/convertase is for example as defined in any one of claims 15-17.

19. A pharmaceutical composition (e.g. a vaccine) comprising the receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of claims 15-18.

20. The receptor/convertase (or homologue, derivative, allelic form, species variant, mutein or equivalent) or pharmaceutical composition as defined in any one of claims 15-19 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

21. An antibody (or antibody derivative) specific for the receptor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in any one of claims 15-20.

22. The antibody of claim 21 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

23. An RP-factor antagonist or inhibitor.

24. The antagonist or inhibitor of claim 23 which comprises:
(a) the antibody of claim 13, 14, 21 or 22; and/or
(b) the receptor of claims 15-20; and/or
(c) an RP-factor mutein which comprises an altered RP-factor specificity-determining domain or which lacks a functional signalling domain.

25. The antagonist or inhibitor of claim 23 or 24 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form or in a form suitable for local or systemic administration.

26. An RP-factor agonist, activator or mimetic.

27. The agonist, activator or mimetic of claim 26 which comprises:
(a) the antibody of claim 21 or 22; and/or
(b) an RP-factor mutein comprising (or consisting of) an RP-factor specificity-determining domain; and/or
(c) an RP-factor mutein comprising (or consisting of) an RP-factor signalling domain; and/or
(d) an RP-factor convertase; and/or
(e) operably coupled combinations of any of (a)-(d).

28. The agonist, activator or mimetic of claim 27 which is:
(a) for use in therapy (e.g. immunotherapy), diagnosis or prophylaxis; and/or
(b) in a pharmaceutical excipient, a unit dosage form, in a form suitable for local or systemic administration or in admixture with an antibiotic.

29. The agonist, activator or mimetic of claim 28 which is for use in adjunctive therapy (for example in combination with an antibiotic).

30. Isolated nucleic acid encoding the RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent thereof) or RP-factor receptor as defined in the preceding claims.

31. A vector (e.g. an expression vector) comprising the nucleic acid of claim 30.

32. A host cell comprising the vector of claim 31.

33. A culture or transport medium comprising an RP-factor (e.g. the factor (or homologue, derivative, allelic form, species variant, mutein or equivalent) as defined in the preceding claims), for example comprising a culture supernatant containing an RP-factor.

34. A nucleic acid probe comprising nucleic acid complementary to the nucleic acid of claim 30.

35. A diagnostic kit comprising an RP-factor (or homologue, derivative, allelic form, species variant, mutein or equivalent), receptor, antibody, probe, culture supernatant or culture medium as defined in any one of the preceding claims.

36. Antisense DNA corresponding to the nucleic acid of claim 30.

37. A process for producing an antimicrobial drug comprising the steps of:
(a) providing an RP-factor receptor;
(b) providing candidate drugs;
(c) screening the candidate drugs by contacting the RP-factor receptor with one of the candidate drugs and determining the affinity of the candidate drug for the RP-factor receptor, wherein the affinity is an index of antimicrobial activity, and optionally
(d) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (c).

38. A process for producing an antimicrobial drug comprising the steps of:
(a) providing an RP-factor receptor;
(b) providing a candidate drug;
(c) providing an RP-factor;
(d) screening the candidate drugs by contacting the RP-factor receptor with one of the candidate drugs in the presence of the RP-factor, and then determining the ability of the candidate drug to compete non-productively with the RP-factor for binding to the RP-factor receptor, wherein the competitive binding ability is an index of antimicrobial activity, and optionally
(e) synthesising or purifying a drug having antimicrobial activity on the basis of the identity of the candidate drug screened in step (d).

39. An antimicrobial drug produced by (or obtainable by) the process of claim 37 or 38, or a derivative thereof.

40. A method for determining the microbiological quality of a product (e.g. a foodstuff, pharmaceutical preparation or medical product) comprising the step of contacting a sample of the product with an RP-factor (for example, an RP-factor as defined in the preceding claims).

41. A method of culturing bacterial (e.g. mycobacterial) cells, comprising the step of incubating the cells in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims).

42. An *ex vivo* method of diagnosis, comprising the step of contacting a biological sample with an RP-factor (for example, an RP-factor as defined in the preceding claims).

43. The method of claim 42 wherein the biological sample is incubated with a culture or transport medium as defined in claim 33.

44. A method of:
(a) stimulating the growth of a microorganism; and/or
(b) resuscitating a dormant, moribund or latent microorganism;
comprising the step of contacting the microorganism with an RP-factor (for example, an RP-factor as defined in the preceding claims).

45. A process for producing the RP-factor or RP-factor receptor of the invention comprising the steps of:

(a) culturing the host cell of claim 32, and

(b) purifying the factor or receptor from the cultured host cells (e.g. from a culture supernatant or cell fraction).

46. A process for producing the RP-factor or receptor of the invention comprising the steps of:

(a) probing a gene library with a nucleic acid probe which is selectively hybridizable with the nucleic acid of claim 30 to produce a signal which identifies a gene that selectively hybridises to the probe;

(b) expressing the gene identified in step (a) (for example by cloning into a host cell, e.g. according to a process as defined in claim 45) to produce the factor or receptor.

47. An RP-factor or receptor obtainable by the process of claim 45 or 46.

48. A process for producing a library of biomolecules comprising the steps of:
(a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived);
(b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture;
(c) isolating microorganisms from the culture of step (b).

49. The process of claim 48 further comprising the step of screening the isolated microorganisms for those which elaborate one or more biomolecules of interest (for example a metabolite, enzyme, antibiotic (e.g. antiviral, antibacterial or antifungal agent) or toxin).

50. A biomolecule produced by (or obtainable by) the process of claim 48 or 49, or a derivative thereof.

51. A process for producing a library of microorganisms (e.g. bacteria) comprising the steps of:
(a) providing a sample (e.g. a soil, marine, food, freshwater, tissue or organism-derived sample);

(b) incubating the sample in a culture medium comprising an RP-factor (for example, an RP-factor as defined in the preceding claims or a culture supernatant comprising an RP-factor) to produce a microbial culture;
(c) isolating microorganisms from the culture of step (b); and optionally
(d) culturing and/or mutagenising the microorganism.

52. A microorganism produced by (or obtainable by) the process of claim 51, or a derivative (e.g. mutant) thereof.

53. Use of a culture supernatant (or fraction or extract thereof) containing an RP-factor for:
(a) diagnosis, prophylaxis or therapy; or
(b) producing a library of microorganisms (e.g. according to the method of claim 51); or
(c) producing a library of biomolecules (e.g. according to the method of claim 48); and/or
(d) resuscitating a dormant, moribund or latent pathogen (e.g. according to the method of claim 44(b)).

54. A culture supernatant (or fraction or extract thereof) containing an RP-factor for use in therapy, prophylaxis or diagnosis.

55. An *ex vivo* method of diagnosis comprising the step of incubating a sample with a culture supernatant (or fraction or extract thereof) containing an RP-factor (or an RP-factor as defined in any one of the preceding claims) at a concentration sufficient to promote the recovery of microrganisms from the sample by culture.

56. The method of claim 55 wherein the sample:
(i) is from an accessible body site, for example a mucous membrane of the vagina, anus, nose, urethra, cervix, skin, conjunctiva, mouth or throat; and/or
(ii) comprises a fluid or semi-solid (for example a bodily fluid or semi-solid, e.g. discharge, vomit, secretion, excreta, sputum or blood); and/or
(iii) comprises a solid (e.g. stool, tissue, food or biopsy sample); and/or
(iv) comprises a culture (e.g. a microbiological culture).

57. A live vaccine comprising an attenuated microbe, which microbe bears a mutation in a gene encoding (or regulating the expression or activity of) one or more RP-factors.

58. The vaccine of claim 57 wherein the microbe selected from any of: an actinomycete, mycobacterium (for example *M. tuberculosis, M. leprae, M. bovis* (e.g. *M. bovis* BCG) and *M. avium*), *Corynebacterium* spp. (e.g. *Corynebacterium diphtheriae*), *Tropheryma whippelii, Nocardia* spp. (e.g. *Nocardia asteroides* and *Nocardia brasiliensis*), *Streptomyces* spp. (e.g. *Streptomyces griseus, Streptomyces paraguayensis* and *Streptomyces somaliensis*), *Actinomadura* spp., *Nocardiopsis* spp., *Rhodococcus* spp., *Gordona* spp., *Tsukamurella* spp. and *Oerskovia* spp., a pathogenic high G+C Gram-positive bacterium and a pathogenic low G+C Gram-positive bacterium (for example *Streptococcus* spp., *Staphylococcus* spp., *Listeria* spp., *Bacillus* spp., *Clostridium* spp.

and *Lactobacillus* spp.).

59. The vaccine of claim 57 or claim 58 wherein the mutation is selected from any of: frameshift, deletion, insertion and/or substitution mutations.

60. The vaccine of any one of claims 57-59 wherein the mutation:
(a) comprises a null mutation (e.g. a non-reverting null mutation); and/or
(b) prevents growth of the microbe; and/or
(c) results in the expression of a mutant RP-factor having altered specificity (e.g. in which the specificity-determining domain has been mutated or modified) and/or which lacks a functional signalling domain.

FIG. 1A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Mtubz94752    mlrlvvgalllvlafaggyavaacktvtltvdgtamrvttmksrvidive    50

Mtubz94752    engfsvddrddlypaagvqvhdadtivlrrsrplqisldghdakqvwtta   100

Mtubz94752    stvdealaqlamtdtapaaasrasrvplsgmalpvvsaktvqlndgglvr   150

Mtubz94752    tvhlpapnvaglsaagvpllgsdhvvpaatapivegmgiqvtrnrikkv   200
MtubMTV008    ----------------mpvgwlwrartakgttlknarttliaaaiagt    32

Mlepl04666    ------------------------------------------msesyrkl     8
MtubMTV043    ------------------------------------------msqrhrkpt    9
Mtubz94752    terlplppnarrvedpemnmsrevvedpgvpgtqdvtfavaevnqvetgr   250
Mlutz96935    ------------------------------------------mtlfttsat    9
MlepL01095    ------------------------------------------mpgemldvrklc  12
MtubU38939    ----------mhplpadhgrsrcnrhpispslsliqnisatsqdmssmt    38
Mtubz81368    ------------------------------mtpgllttagagrprdrca    19
MtubMTV008    lvttspagianaddagldpnaaagpdavgfdpnlppapdaapvdtppape   82

Scoeli6C12$   ---irtaavtlvaatalgatgeavaapsaplrtDWDAIAACESSGNWQAN    25
Mlepl04666    ttssiivakitftqamldgsialaggaspatdsEWDQVARCESSGGNWSIN   58
MtubMTV043    tsnvsvakiaftqavlgqggiamaaqataatdgEWDQVARCESSGGNWSIN   59
Mtubz94752    lpvanvvvtpaheavvrvgtkpgtevppvidgsIWDAIAGCEAGGNWAIN   300
Mlutz96935    rsrratasivaqmtlagaaavgfsapagaatvdTWDRLAECESNGTWDIN   59
MlepL01095    klfvksavvsgivtasmalststqmanavprePNWDAVAQCESGRNWRAN   62
MtubU38939    riakpliksamaaqlvtasmslstavahagpsPNWDAVAQCESGGNWAAN   88
Mtubz81368    rivctvfietavvatmfvallglstisskaddIDWDAIAQCESGGNWAAN   69
MtubMTV008    dagfdpnlppplapdflsppaeeappvpvaysVNWDAIAQCESGGNWSIN   132
                                                ***.* ******* *

Scoeli6C12$   TGNGYYGGLQFARSSWIAAGGLKYAPRADLATRGEQIAVAERLARLQGMS    75
Mlepl04666    TGNGYLGGLQFSQGTWASHGGGEYAPSAQLATREQQIAVAERVLATQGSG   108
MtubMTV043    TGNGYLGGLQFTQSTWAAHGGGEFAPSAQLASREQQIAVGERVLATQGRG   109
Mtubz94752    TGNGYYGGVQFDQGTWEANGGLRYAPRADLATREEQIAVAEVTRLRQGWG   350
Mlutz96935    TGNGFYGGVQFTLSSWQAVGGEG---YPHQASKAEQIKRAEILQDLQGWG   106
MlepL01095    TGNGFYGGLQFKPTIWARYGGVG---NPAGASREQQITVANRVLADQGLD   109
MtubU38939    TGNGKYGGLQFKPATWAAFGGVG---NPAAASREQQIAVANRVLAEQGLD   135
Mtubz81368    TGNGLYGGLQISQATWDSNGGVG---SPAAASPQQQIEVADNIMKTQGPG   116
MtubMTV008    TGNGYYGGLQFTAGTWRANGGSG---SAANASREEQIRVAENVLRSQGIR   179
              ****.******   .* . **          *.***** *** ..  **

Scoeli6C12$   AW----------------------------------------------------
Mlepl04666    AWPACGHGLSGPSLQEVLPAG--MGAPw----INGAPAPLAPPPPAEPAP   152
MtubMTV043    AWPVCGRGLSNATPREVLPASaaMDAPldaaaVNGEPAPLA-PPPADPAP   158
Mtubz94752    AWPVCAaragar--------------------------------------   362
Mlutz96935    AWPLCSQKLgltqadadagdvdateaapvavertatvqrqsaadeaaaeq   156
MlepL01095    AWPKCGAASDLPITLWSHPAQGVKQIINDIIqmgdttlaaialnql----   155
MtubU38939    AWPTCGAASGLPIALWSKPAQGIKQIINEIIwagigasipr----------   176
Mtubz81368    AWPKCSscsqgdaplgslthiltflaaetggcsgsrdd-------------   154
MtubMTV008    AWPVCGrrg----------------------------------------   188
              *** *.
```

FIG. 1A (CONT.)

```
Mlep104666      pqppadnf----------PPTPGDVPSPLarp-----   174
MtubMTV043      pvelaandlpaplgeplpaapadpappadlaPPAPADVAPPVelavndlp   208
MlutZ96935      aaaaeqavvaeaetivvksgdslwtlaneyeveggwtalyeankgavsda   206

MtubMTV043      aplgeplpaapadpappadlappapadlappapadlappapadlappvel   258
MlutZ96935      aviyvgqelvlpqa----------------------------------   220

MtubMTV043      avndlpaplgeplpaapaelappadlapasadlappapadlappapaela   308

MtubMTV043      ppapadlappaavneqtapgdqpatapggpvglatdlelpepdpqpadap   358

MtubMTV043      ppgdvteapaetpqvsniaytkklwqairaqdvcgndaldslaqpyvig-   407
```

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FIG. 1B

```
A  YabEBsubt   mgeregrvdslldtlynlseekeaffitqkmkklfsvklsKSKVILVAACLLLAGSGTAYAAHELTKQSVSVSINGKKKHIR   82
A  MtubZ94752  ----------------------------------------MLRLVVGALLLVLAFAGG-YAVAACKTVTLTVDGTAMR--VT   39

A  YocHBsubt   ----------------------------------------------------------------------mkktimsfvav   11
A  YabEBsubt   THANTVGDLLETLDIKTRDEDKITPAKQTKITADMDVVYEAAKPVKLTING-EEKTLWSTAKTVGALLDEQDVDVKEQDQID  163
A  MtubZ94752  TMKSRVIDIVEENGFSVDDRDDLYPAAGVQVHDADTIVLRRSRPLQISLDGHDAKQVWTTASTVDEALAQLAMTDTAPAAAS  121
A  Caceto506   -------------------KR??AVILMVAVIFTIISSMKKNITVNIDG-KTSKIITYKSNEGSILSKNNILVGPKDKIQ   58
                             #     .     ..    . . . ..#        .     .  #    .

A  yocHBsubt   aalsttafgahasakeitvqkgdtlwgisqkngvnlkdlkewnkltsdkiiagekltisseettttgqytikagdtlskiaq   93
A  YabEBsubt   PAIDTDISKDMKINIEPAFQVTVNDAGKQKKIWTTSTTVADFLKQQKMNIKDEDKIKPALDAKLTKGKAD-ITITRIEKVTD  244
A  MtubZ94752  RASRVPLSG-MALPVVSAKTVQLNDGGLVRTVHLPAPNVAGLLSAAGVPLLQSDHVVPAATAPIVEGMQIQVTRNRIKKVTE  202
A  Caceto506   PALDTNLKNGDKIYIKKAISVEVAVDGKVRRVKSSEETVSKMLKAEKIPLSKVDKVNISRNAAIKKNM--KISITRVNSQIT  138
               #     .     . .  #  # .   #  . .       #  .#     . .    # .     # .      .. #.

A  yocHBsubt   kfgttvnnlkvwnnlssdmiyagstlsvkgqataantatenaqtnapqaapkqeavqkeqpkqeavqqqpkqetkaeaetsv  175
A  YabEBsubt   VVEEKIAFDVKKQEDASLEKGKEKVVQKGKEGKLKKHFEVVKENGKEVSRELVKEETAEQSKDKVIAVGTKQSSPKFETVSA  326
A  MtubZ94752  RLP--LPPNARRVEDPEMNMSREVVEDPGVPGTQDVTFAVAEVNGVETGRLPVANVVVTPAHEAVVRVGTKPGTEVPPVIDG  282
A  Caceto506   KENQQVDFPTEVISDDSMGNDEKQVIQQGQAGEKEVFTKIVYEDGKAVSKEIVGEVIKKEPTKQVFKVGTlgvlkpdrggrv  220
                    .        #        # . #  #       .    .#    .  #                 #..###

B  MtubZ94752  Siwdaiagceaggnwaintgngyyggvqfdqgtweangglryapradlatreeqiavaevtrlrqgwgawpvcaaragar--  362
B  yocHBsubt   NTEEKAVQSNTNNQEASKELTVTATAYTANDGGISGVTATGIDLNKNPNA-KVIAVDPNVIPLGSKVYVEGYGEATTAADTG  256
B  YabEBsubt   SGDSKTVVSRSNE-STGKVMTVSSTAYTASCSGCSGHTATGVNLKNNPNA-KVIAVDPNVIPLGSKVHVEGYGYAIIAADTG  406
B  Caceto506   ---------------LYKKSLQVLATAYTDDFSF--GITASGTKVKRDSDGYSSIAVDPTVIPLGTKLYVPGYGYGVVAEDTG  286
B  Cperfring   -----------------------AEAYTA----------SGMHVLRDPNGYSTIAVDPSVIPLGTKLYVEGYGYAIIAADTG   49
                                   * . *  *****      * **.*   .  .  ..    ***** *****.*.********..*****

B  YocHBsubt   GAIKGNKIDVFVPEKSSAYRWGNKTVKIKILN  288
B  YabEBsubt   SAIKGNKIDVFFPSKSDASNWGVKTVSVKVLN  438
B  Caceto506   GAIKGNRLDLFFTSERECYDWGAKNVTVYILK  318
B  Cperfring   GAIKGNRVDLFFNTEAEASNWGVRNLDVYILN   81
               ******..*.** *  .* .**  . . . .**
```

```
RPF         TIVVKSGDSLWTLANE-YEVEGGWTALYEANKGAVS----------DAAVIYVGQELVLPQA
g149657     TIKVKSGDSLWKLSRQ-YDTT--ISALKSENKL-------------KSTVLYVGQSLKVPES
g2226145    TIKVKSGDSLWKLAQT-YNTS--VAALTSANHL-------------STTVLSIGQTLTIP--
g2226145    TYTVKSGDSLWVIAQK-FNVT--AQQIREKNNL-------------KTDVLQVGQKLVI---
g2226145    KYTVKSGDSLWKIANN-INLT--VQQIRNINNL-------------KSDVLYVGQVLKL---
g266725     TYTVKSGDTIWALSSK-YGTS--VQNIMSWNNL-------------SSSSIYVGQVLAVKQ-
g80581      THAVKSGDTIWALSVK-YGVS--VQDIMSWNNL-------------SSSSIYVGQKLAIKQ-
g2707292    SVKVKSGDTLWALSVK-YKTS--IAQLKSWNHL-------------SSDTIYIGQNLIVSQS
g755216     TYTVKSGDTLWGISQR-YGIS--VAQIQSANNL-------------KSTIIYIGQKLLL---
g1722873    TYTVKKGDTLWDIAGRFYGNSTQWRKIWNANKTAMIKRSKRNIRQPGHWIFPGQKLKIPQ-
g1176755    TYTVKKGDTLWDLAGKFYGDSTKWRKIWKVNKKAMIKRSKRNIRQPGHWIFPGQKLKIPQ-
            .  **.** .*         .       .    * .                 .  ** *
```

FIG. 1C

```
1 msgrhrkpttsnvsvakiaftgavlggggiamaaqataatdgewdqvarcesggnwsintgngylgg
  lqftqstwaahgggefapsaqlasreqqiavgervlatqgrgawpvcgrglsnatprevlpasaamd
  apldaaavngepaplapppadp 156

157 appvelaandlpaplgeplpaapadpappadlappapadv 196
197 appvelavndlpaplgeplpaapadpappadlappapadlappapadlappapadl 252
253 appvelavndlpaplgeplpaapaelappadlap-asadlappapadlappapaelappapadlappa
320 ------avne 323

324 qtapgdqpatapggpvglatdlelpepdpqpadapppgdvteapaetpqvsniaytkklwqaira
389 qdvcgndaldslaqpyvig* 407
```

Motif	sequence	
A	157 appvelaandl	167
B'	168 paplgeplpaapad	181
C	182 pappadl	188
D	189 appapadv	196
A	197 appvelavndl	207
B'	208 paplgeplpaapad	221
C	222 pappadl	228
D	229 appapadl	236
D	237 appapadl	244
D	245 appapadl	252
A	253 appvelavndl	263
B	264 paplgeplpaapael	278
'C	279 appadl	284
D*	285 apasadl	291
D	292 appapadl	299
D	300 appapael	307
D	308 appapadl	315
D'	316 appa	319
'A'	320 avne	323

A = appvela[av]ndl

B = paplgeplpaapa[de]l

C = pappadl

D = appapa[de][lv]

Fig. 1D

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FIG. 1E

```
  1 accaaggagaaggacgaccccggtgtgcctcggccgccgatcagcgaggactcgccatgg 60
 61 acaccatgactctcttcaccacttccgccacccgctccgccgtgccaccgcctcgatcg 120
       M  T  L  F  T  T  S  A  T  R  S  R  R  A  T  A  S  I  V
                                                              g
121 tcgcgggcatgaccctcgccggcgccgccgccgtgggcttctccgccccggcccaggccg 180
     A  G  M  T  L  A  G  A  A  A  V  G  F  S  A  P  A  Q  A  A
                                                              A

                          oligo A1>>>
   csacsgtsgacacstgggaccgsctsgcsgag
181 ccaccgtggacacctgggaccgcctcgccgagtgcgagtccaacggcacctgggacatca 240
     T  V  D  T  W  D  R  L  A  E  C  E  S  N  G  T  W  D  I  N
     T  V  D  T  W  D  R  L  A  E  E  x  S  N  G  T  x  D

         <<< oligo G2                    oligo G1>>>
         gttgccgaagatgccgcc      agttcaccctgtcctcctg
241 acaccggcaacggcttctacggcggcgtgcagttcaccctgtcctcctggcaggccgtcg 300
     T  G  N  G  F  Y  G  G  V  Q  F  T  L  S  S  W  Q  A  V  G
                                                           V  G

     <<< oligo A2
     ccictycciatrggigtrgtycg
301 gcggcgaaggctacccgcaccaggcctcgaaggccgagcagatcaagcgcgccgagatcc 360
     G  E  G  Y  P  H  Q  A  S  K  A  E  Q  I  K  R  A  E  I  L
     G  E  G  Y  P  H  Q  A  S  K
361 tccaggacctgcaggggctggggcgcgtggccgctgtgctcgcagaagctgggcctgaccc 420
     Q  D  L  Q  G  W  G  A  W  P  L  C  S  Q  K  L  G  L  T  Q
421 aggctgacgcggacgccggtgacgtggacgccaccgaggccgccccggtcgccgtggagc 480
     A  D  A  D  A  G  D  V  D  A  T  E  A  A  P  V  A  V  E  R
481 gcacggccaccgtgcagcgccagtccgccgcggacgaggctgccgccgagcaggccgctg 540
     T  A  T  V  Q  R  Q  S  A  A  D  E  A  A  A  E  Q  A  A  A
541 ccgcggagcaggccgtcgtcgccgaggccgagaccatcgtcgtcaagtccggtgactccc 600
     A  E  Q  A  V  V  A  E  A  E  T  I  V  V  K  S  G  D  S  L
601 tctggacgctcgccaacgagtacgaggtggagggtggctggaccgccctctacgaggcca 660
     W  T  L  A  N  E  Y  E  V  E  G  G  W  T  A  L  Y  E  A  N
661 acaagggcgccgtctccgacgccgccgtgatctacgtcggccaggagctcgtcctgccgc 720
     K  G  A  V  S  D  A  A  V  I  Y  V  G  Q  E  L  V  L  P  Q
721 aggcctgagacgcctgaccggccccccggaccggtacc 758
     A  *
```

```
1   ATVDTWDRLA ECESNGTWDI NTGNGFYGGV QFTLSSWQAV GGEGYPHQAS KAEQIKRAEI     60
61  LQDLQGWGAW PLCSQKLGLT QADADAGDVD ATEAAPVAVE RTATVQRQSA ADEAAAEQAA    120
121 AAEQAVVAEA ETIVVKSGDS LWTLANEYEV EGGWTALYEA NKGAVSDAAV IYVGQELVLP QA 182
```

FIG. 2A

```
ggatccgcaccgccgcggtaaccctggtcgccgcgaccgcactcggggcgaccggcgaag  60
  I  R  T  A  A  V  T  L  V  A  A  T  A  L  G  A  T  G  E  A

cggtggccgcgccctcggcgcccctgcgcaccgactgggacgccatcgccgcgtgcgagt 120
  V  A  A  P  S  A  P  L  R  T  D  W  D  A  I  A  A  C  E  S

ccagcggcaactggcaggcgaacaccggcaacggctactacggcggcctgcagttcgcac 180
  S  G  N  W  Q  A  N  T  G  N  G  Y  Y  G  G  L  Q  F  A  R

ggtccagctggatcgccgccggcggcctcaagtacgccccgcgcgcggacctcgccaccc 240
  S  S  W  I  A  A  G  G  L  K  Y  A  P  R  A  D  L  A  T  R

gcggcgagcagatcgccgtggcggaacgcctcgcccgtctgcaggggatgtccgcctgg  299
  G  E  Q  I  A  V  A  E  R  L  A  R  L  Q  G  M  S  A  W
```

FIG. 2B

FIG. 3

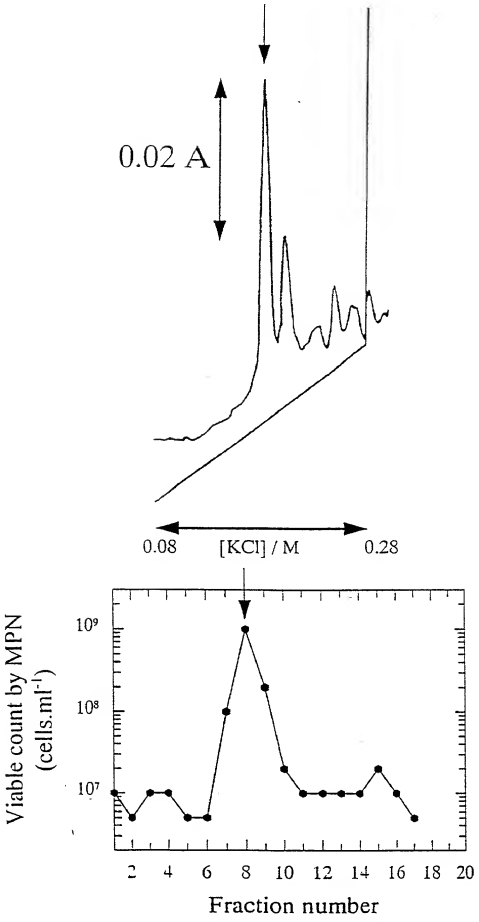
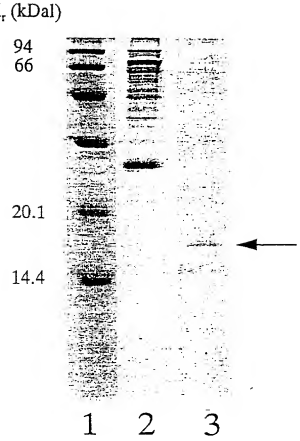

A
0.02 A
B
0.08
[KCl] / M
0.28
Viable count by MPN
(cells.ml-1)
10⁹
10⁸
10⁷
2
4
6
8
10
12
14
16
18
20
Fraction number

FIG. 3

C.


M$_r$ (kDal)
94
66
20.1
14.4
1
2
3

D.

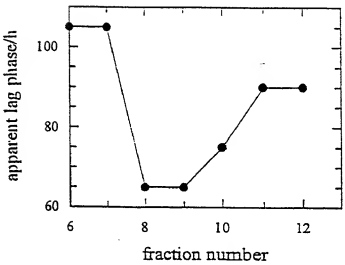
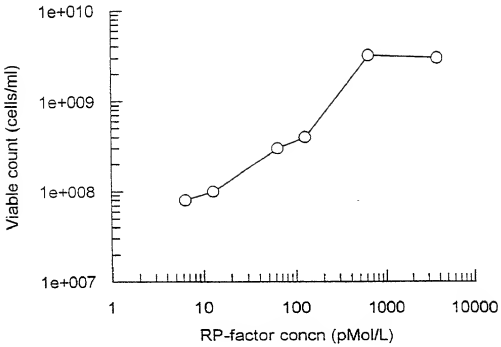
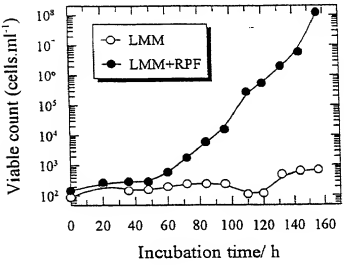

apparent lag phase/h
100
80
60
6
8
10
12
fraction number

FIG. 4

A

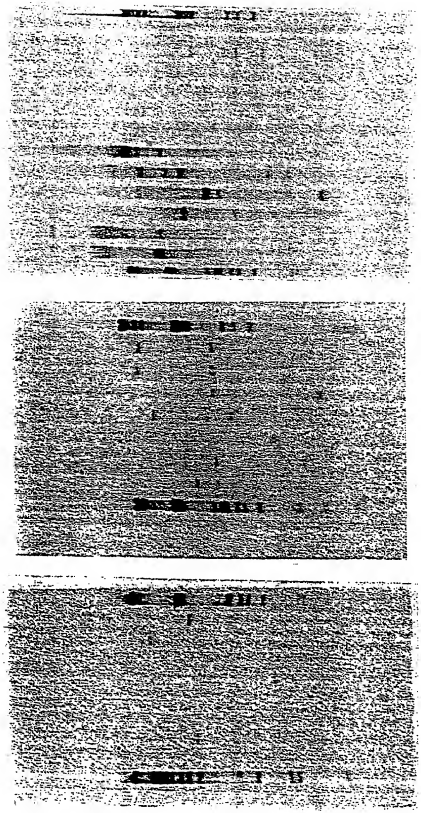

1e+010
1e+009
1e+008
1e+007
Viable count (cells/ml)
1
10
100
1000
10000
RP-factor concn (pMol/L)

B


LMM
LMM+RPF
Viable count (cells.ml^{-1})
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0 20 40 60 80 100 120 140 160
Incubation time/ h

12/20

FIG.5



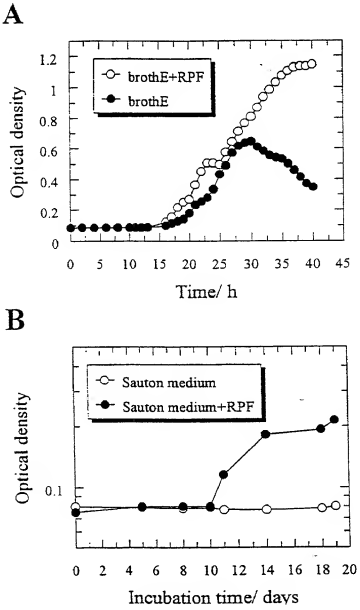

A
brothE+RPF
brothE
Optical density
1.2
1
0.8
0.6
0.4
0.2
0
0 5 10 15 20 25 30 35 40 45
Time/ h
B
Sauton medium
Sauton medium+RPF
Optical density
0.1
0 2 4 6 8 10 12 14 16 18 20
Incubation time/ days

FIG. 6

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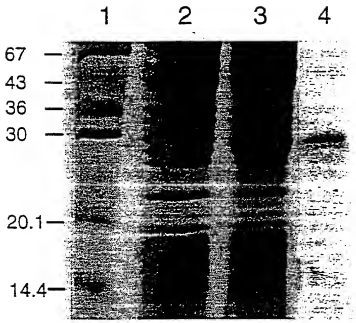
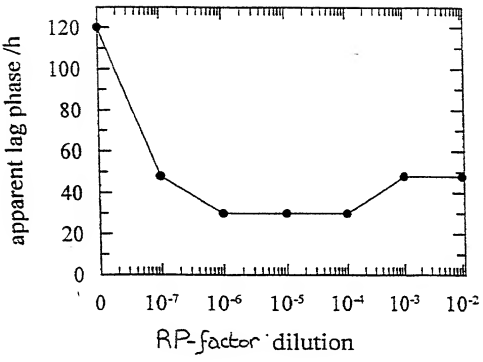
FIG. 7

A


1
2
3
4
67
43
36
30
20.1
14.4

B


apparent lag phase /h
120
100
80
60
40
20
0
0
10-7
10-6
10-5
10-4
10-3
10-2
RP-factor dilution

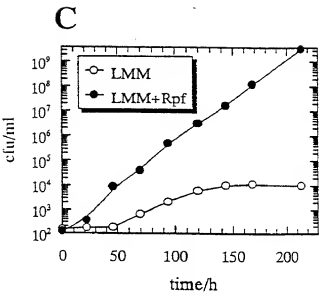

C
LMM
LMM+Rpf
cfu/ml
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
50
100
150
200
time/h

FIG. 7

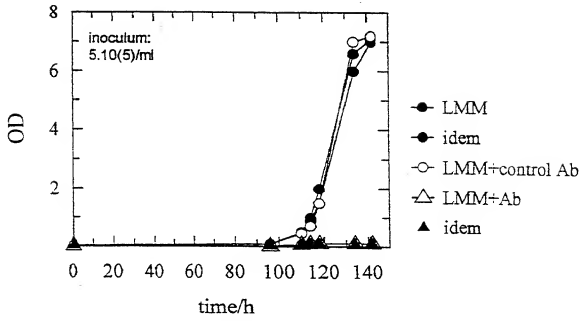
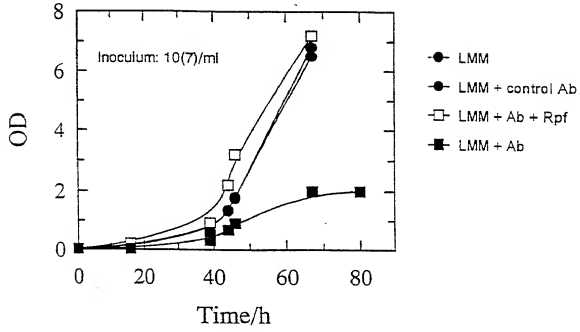

inoculum:
5.10(5)/ml
OD
8
6
4
2
0 20 40 60 80 100 120 140
time/h
LMM
idem
LMM+control Ab
LMM+Ab
idem

FIG. 8A

FIG. 8 B



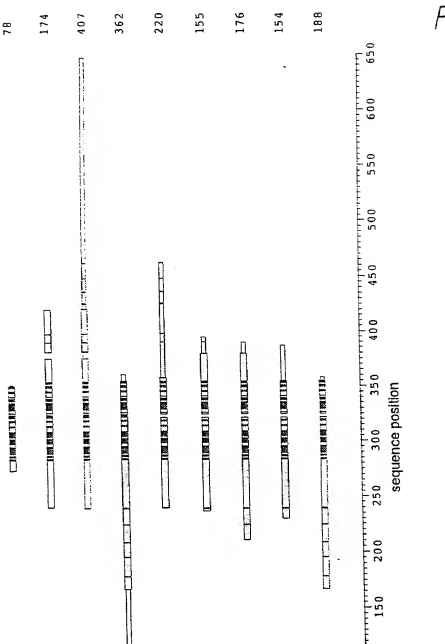

ScoalIcolor
MlepL04666 -
MtubMTV043
Mtub294752
MlutFactor
MlepL01095
MtubU38939
Mtub281368
MtubMTV008
78
174
407
362
220
155
176
154
188
0
50
100
150
200
250
300
350
400
450
500
550
600
650
sequence position

FIG. 9A

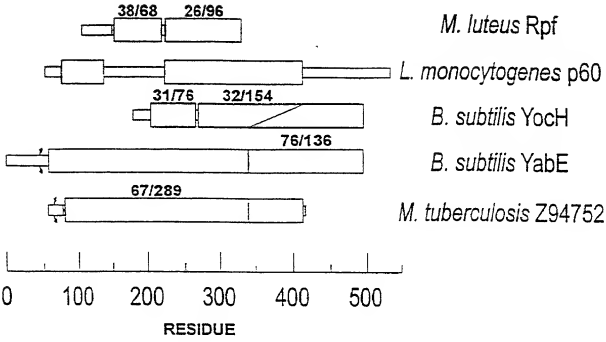
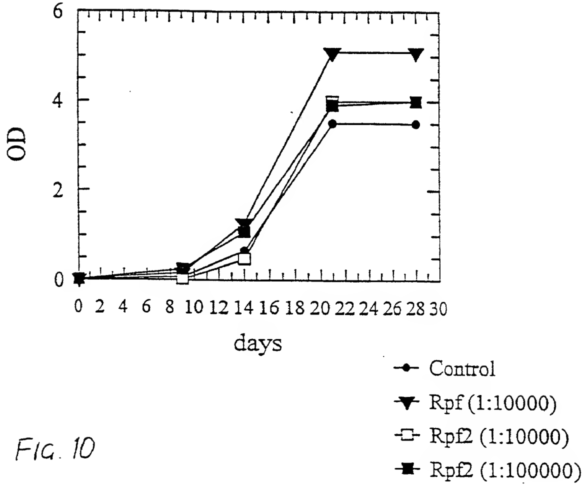

38/68
26/96
M. luteus Rpf
L. monocytogenes p60
31/76
32/154
B. subtilis YocH
76/136
B. subtilis YabE
67/289
M. tuberculosis Z94752
0
100
200
300
400
500
RESIDUE

FIG. 9B



6
4
2
0
OD
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
days
Control
Rpf (1:10000)
Rpf2 (1:10000)
Rpf2 (1:100000)

FIG. 10

P573.98P

Customer Number: 000959

Attorney's Docket Number FHW-051US

Declaration, Petition and Power of Attorney for Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

BACTERIAL PHEROMONES AND USES THERFOR

the specification of which

(check one)

_ is attached hereto.

_ was filed on _____ as

Application Serial No. _____

and was amended on _____.
(if applicable)

I do not know and do not believe that the subject matter of this application was known or used by others in the United States or patented or described in a printed publication in any country before my invention thereof, or patented or described in a printed publication in any country or in public use or on sale in the United States more than one year prior to the date of this application, or first patented or caused to be patented or made the subject of an inventor's certificate by me or my legal representatives or assigns in a country foreign to the United States prior to the date of this application on an application filed more than twelve months (six months if this application is for a design) before the filing of this application; and I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application, that no application for patent or inventor's certificate on the subject matter of this application has been filed by me or my representatives or assigns in any country foreign to the United States, except those identified below, and that I have reviewed and understand the contents of the specification, including the claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

CLAIM OF BENEFIT OF EARLIER FOREIGN APPLICATION(S)

I hereby claim priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below, and have also identified below any foreign application(s) for patent or inventor's certificate filed by me on the same subject matter having a filing date before that of the application(s) from which priority is claimed.

Check one:

X no such applications have been filed.

_ such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (month,day,year)	Priority Claimed Under 35 USC 119
Great Britain	9711389.8	06.04.1997	x Yes No _
Great Britain	9811221.2	05.27.1998	x Yes No _
			_ Yes No _
			_ Yes No _
			_ Yes No _

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

CLAIM FOR BENEFIT OF U.S. PROVISIONAL APPLICATION(S)

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

____________________ ____________________
(Application Serial No.) (Filing Date)

____________________ ____________________
(Application Serial No.) (Filing Date)

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of any earlier United States application(s) or PCT international application(s) designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the earlier application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date(s) of the earlier application(s) and the national or PCT international filing date of this application. As to subject matter of this application which is common to my earlier application(s), if any, described below, I do not know and do not believe that the same was known or used by others in the United States or patented or described in a printed publication in any country before my invention thereof, or patented or described in a printed publication in any country or in public use or on sale in the United States more than one year prior to the date(s) of said earlier application(s), or first patented or caused to be patented or made the subject of an inventor's certificate by me or my legal representatives or assigns in a country foreign to the United States prior to the date(s) of said earlier application(s) on an application filed more than twelve months (six months if this application is for a design) before the filing of said earlier application(s); and I acknowledge that no application for patent or inventor's certificate on said subject matter has been filed by me or my representatives or assigns in any country foreign to the United States except those identified herein.

PCT/GB98/01619	3rd JUNE 1998	PENDING
(Application Serial No.)	(Filing Date)	(Status) (patented,pending,aband.)
(Application Serial No.)	(Filing Date)	(Status) (patented,pending,aband.)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

W. Hugo Liepmann	Reg. No. 20,407	Lawrence E. Monks	Reg. No. 34,224
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Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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